MOLECULAR BIOLOGY OF THE RESPIRATORY NADH DEHYDROGENASE OF *ESCHERICHIA COLI*

by

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at the Australian National University, 1983.

STATEMENT

All the experimental work reported in this thesis was performed by the author, unless specifically stated otherwise in the text.

Bruce Lee Rogers.

B.L.Rogers

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PREFACE

This thesis describes the results of research carried out in the Department of Biochemistry, John Curtin School of Medical Research, Australian National University.

Abbreviations used in this thesis without definition include:

kb	kilobase pairs
bp	base pairs
mol wt	molecular weight
M_r	apparent molecular weight
FAD	flavin-adenine dinucleotide
Tris HCl	tris(hydroxymethyl)aminomethane hydrochloride
EDTA	ethylenediaminetetraacetate
b.p.	boiling point
SDS	sodium dodecyl sulphate

The following enzyme nomenclature has been adopted: NADH dehydrogenase (1.6.99.1), any enzyme (complex) which catalyzes the oxidation of NADH by artificial electron acceptors; Respiratory NADH dehydrogenase, the enzyme (complex) which catalyzes the transfer of electrons between NADH and ubiquinone *in vivo*. In the case of the purified respiratory NADH dehydrogenase of *E. coli* described in this thesis, it is also referred to as the NADH:ubiquinone oxidoreductase to signify that ubiquinone-1 is used in assays as the electron acceptor.

NADH oxidase refers to the activity of the intact respiratory chain in oxidizing NADH and reducing endogenous ubiquinone-8 present in *E. coli* membranes. Where enzymes that catalyze specific reactions are described, they are named according to I.U.B. rules, eg NADH:ubiquinone oxidoreductase (1.6.5.3), etc.

ABSTRACT

The work described in this thesis is part of a multidisciplinary approach to the isolation and characterization of the respiratory NADH dehydrogenase of E.coli.

The complete nucleotide sequence for the ndh structural gene was determined using the Sanger chain-termination method (Young, I.G., Rogers, B.L., Campbell, H.D., Jaworowski, A., and Shaw, D.C. (1981) Eur. J. Biochem. 116, 165-170). Analysis of the sequence revealed the following features: (a) A putative promoter approximately 40 bp upstream from the initiation codon, (b) two inverted repeats, one upstream from the initiation codon (covering sections of the putative promoter), and the other immediately after the stop codon. The latter repeat is believed to act as an RNA transcription termination structure. (c) A ribosome binding site, complementary to the 16S ribosomal RNA immediately precedes the initiation codon. (d) UUG, which codes for N-formylmethionine rather than the usual leucine residue acts as an atypical initiation codon.

An examination of the primary amino acid sequence led to the detection of internal homology within the enzyme. There are two large repeating units of ~130 amino acids near the N-terminus of the protein and two smaller repeats of ~20 amino acids near the C-terminus. Comparison of the primary amino acid sequence of the enzyme with other proteins led to the detection of homology with the human glutathione reductase. By comparison with the well known three-dimensional structure of glutathione reductase, the two large N-terminal repeats of the NADH dehydrogenase can be assigned as the FAD and NADH binding domains. The function of the short C-terminal repeats is unknown.

The catalytic activity of the purified E. coli respiratory NADH dehydrogenase was examined with short and long chain homologues of ubiquinone. The purified enzyme catalyzes the reduction of various ubiquinone homologues at high rates without the involvement of any auxilliary protein(s). The enzyme was reconstituted into phospholipid vesicles, after which it possesses high ubiquinone-8 reductase activity. Results of kinetic and inhibition studies are consistent with the view that ubiquinone-1, ubiquinone-3 and ubiquinone-8 all act at the same catalytic site on the enzyme and that ubiquinone-8 is the immediate electron acceptor for the E. coli respiratory NADH dehydrogenase in vivo

The reconstitution of membrane vesicles of ndh mutants by purified respiratory NADH dehydrogenase was investigated. The enzyme can readily become membrane-associated and reconstitute the NADH oxidase of membrane vesicles from ndh mutants. The purified enzyme was shown to be capable of inserting into the outer surface of the inner cytoplasmic membrane of ndh spheroplasts. Once assembled, the enzyme does not readily 'flip' across the lipid bilayer even when amplified to 70-fold wild type levels. The observation that the purified enzyme can reconstitute ndh spheroplasts suggests that there is no obligatory protein receptor that facilitates membrane binding.

A novel [^{14}C]-labelled photoaffinity analogue ([^{14}C]-2'-arylazidoubiquinone-1) of ubiquinone was synthesized in an attempt to probe the ubiquinone binding site of the purified enzyme. After photolysis the [^{14}C]-2'-arylazidoubiquinone-1 was shown to be covalently attached (typically 0.1-0.15 mole label per mole polypeptide) to the purified enzyme. The labelled enzyme was cleaved at tryptophan residues by BNPS-skatole. The labelled polypeptides

produced were separated with high resolution, purified and analyzed. The results suggested that the middle region of the protein appeared to be more heavily labelled than the C-terminal region which was more labelled than the N-terminal region.

With the aim of assisting with the identification of the ubiquinone binding site of the respiratory NADH dehydrogenase, the determination of the DNA sequence of the dld gene (codes for D-lactate dehydrogenase) was initiated. The cloned ~6.9 kb DNA fragment which contains the dld gene was isolated and purified after HindIII digestion of pIY2 (Young, I.G., Jaworowski, A., and Poulis, M.I. (1978) Gene 4, 25-36). Although time did not permit the completion of the sequence, approximately 1030 bases of unique DNA sequence was obtained.

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Chapter 1

General Introduction

The Aerobic Respiratory Chain of E. coli

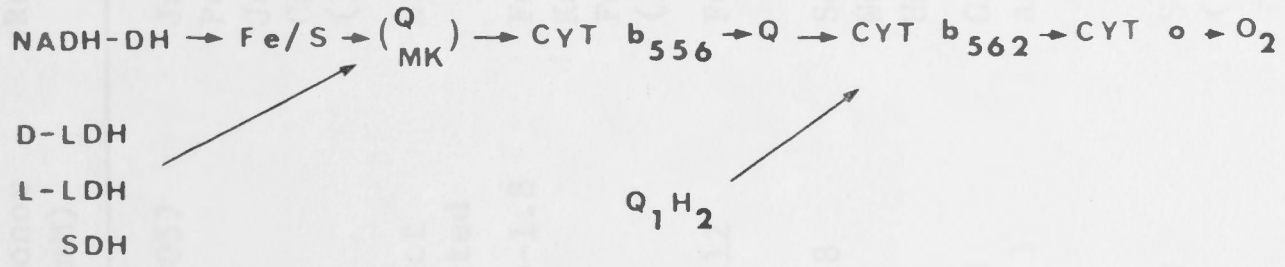
The aerobic respiratory chain of E. coli and mitochondria are similar in their basic design. Both systems possess substrate-specific dehydrogenases which catalyze electron transfer via ubiquinone to a cytochrome chain and ultimately to molecular oxygen.

There have been a number of schemes proposed for the arrangement of the components in the respiratory chain of E. coli (for example Haddock and Jones (1977), Downie and Cox (1978) and Kita, Kasahara and Anraku (1982)). These schemes differ in several respects, most importantly in the site(s) of ubiquinone interaction and the sequence of the cytochromes. One such scheme, taken from Kita and Anraku (1981) is presented in Figure 1-1. However, although the various schemes may be valuable as a stimulus for further experimentation, there is still no general agreement on the order of the respiratory components, and the involvement of iron-sulphur proteins is also uncertain.

Several respiratory-chain-linked dehydrogenases have been purified from the inner membrane of E. coli. Some of their biochemical properties are listed in Table 1-1. Of the enzymes listed in Table 1-1, the best characterized are the NADH dehydrogenase and the D-lactate dehydrogenase.

Under conditions of vigorous aeration the major membrane-bound cytochromes are cytochromes b_{556}, b_{562} and o, whereas under anaerobic growth the major-membrane bound cytochromes are cytochromes b_{556}, b_{558} and a_2 (cytochrome d) together with low concentrations of a_1 (Haddock and Schairer, 1973).

Figure 1-1. Sequence of the Cytochrome b_{556} Electron-Carrying Chain of Aerobically Grown E. coli



This sequence is for aerobically grown E. coli in the early exponential phase. Abbreviations used include: NADH-DH, NADH dehydrogenase; SDH, succinate dehydrogenase; L-LDH, L-lactate dehydrogenase; D-LDH, D-lactate dehydrogenase; Fe/S, iron-sulphur protein; Q, ubiquinone-8; MK, menaquinone-8; cyt, cytochrome. The figure is taken from Kita and Anraku (1981).

Table 1-1. Properties of the Purified Respiratory-Chain-Linked Dehydrogenases of *E. coli*

Enzyme	Mol Wt (monomer)	Prosthetic Group	Specific Activity[a]	Acceptor	K_m Donor (mM)	References
NADH	47000	FAD	500-600	Q-1	0.057	Jaworowski, Campbell, Poulis and Young (1981a); Jaworowski, Mayo, Shaw, Campbell and Young (1981b)
Succinate dehydrogenase	61000	not cited	0.15	not cited	not cited	Bragg (1980)
D-Lactate dehydrogenase	71000 -72000	FAD	200-230[b]	MTT	0.6-1.8	Futai (1973); Kohn and Kaback (1973); Pratt, Fung, Flowers and Ho (1979).
L-Lactate dehydrogenase[c]	43000	FMN	31[b]	MTT	0.12	Futai and Kimura (1977)
Glycerol-3-phosphate dehydrogenase[c]	58000	FAD	37.7	$Fe(CN)_6^{3-}$	0.8	Schryvers, Lohmeier, and Weiner (1978); Weiner and Heppel (1972)
D-Amino acid dehydrogenase[c]	55000 and 45000 (nonidentical subunits)	FAD	3.5 27.0	DCIP Q-1	29 5.3	Olsiewski, Kaczorowski and Walsh (1980)
Proline dehydrogenase[c]	124000	FAD	7.3	p-iodonitro tetrazolium	60	Scarpulla and Soffer (1978)

Table 1-1. (continued)

Enzyme	Mol Wt (monomer)	Prosthetic Group	Specific Activity[a]	Acceptor	K_m Donor (mM)	References
Pyruvate oxidase	66000	FAD	200	$Fe(CN)_6^{3-}$	10	Williams and Hagar (1966); O'Brien, Schrock, Russell, Blake and Gennis (1976)

a μmol (donor) min^{-1} mg^{-1} protein.
b Assayed by phenazine methosulphate (PMS) mediated reduction of MTT (3-(4,5-dimethylthiazolyl-2)-2, 5-diphenyltetrazolium bromide).
c Inducible enzyme.

Both cytochromes o and d have been shown to function as terminal oxidases in the respiratory chain of E. coli (Caster and Chance, 1959; Pudek and Bragg, 1974). The relative amounts of these two cytochromes is altered by changes in growth conditions. Thus, stationary phase cells have a much greater relative amount of cytochrome d to cytochrome o compared to cells harvested in the exponential phase of growth (Pudek and Bragg, 1974; Rice and Hempfling, 1978). Pudek and Bragg (1974) demonstrated that oxidation proceeding via cytochrome o is more sensitive to cyanide than cytochrome d.

Recently, Green and Gennis (1983) reported experiments with an E. coli cytochrome d mutant (cyd) which showed that growth and oxygen consumption are maintained by cytochrome d in the presence of cyanide concentrations which inactivate cytochrome o.

Kita et al. (1982) have purified the b_{562}-o complex (terminal oxidase) and reconstituted the preparation into liposomes. Furthermore, they provided evidence that the cytochrome b_{562}-o complex functions as a coupling site (ibid). They also found that the cytochrome b_{558}-d complex, a major terminal oxidase of E. coli cells in the late exponential phase of aerobic growth serves as a coupling site (ibid).

Interestingly, a terminal oxidase complex which contains cytochrome b_{558}, a_1 and d and only two major polypeptide subunits has recently been purified and reported to function as a ubiquinol-8 oxidase in reconstituted proteoliposomes (Miller, Koland, and Gennis, unpublished data cited in Green and Gennis, 1983).

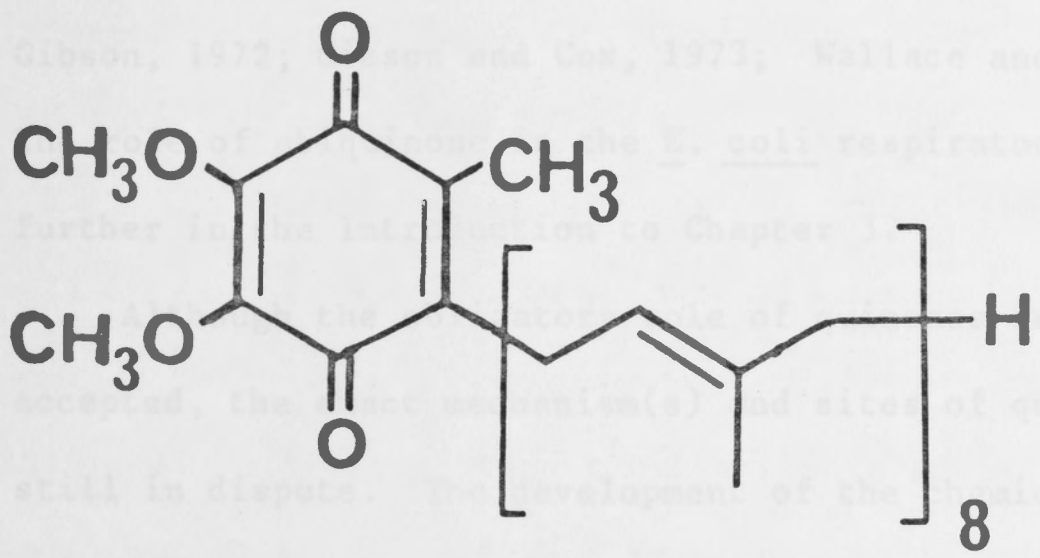
Quinone Function

Quinones are known to play an important role in a number of diverse and essential biological processes. Most notable is their role in the respiratory chain in mitochondria and bacteria (Redfearn, 1966; Brodie and Watanabe, 1966) as well as in photosynthesis in plants (Crane and Henninger, 1966).

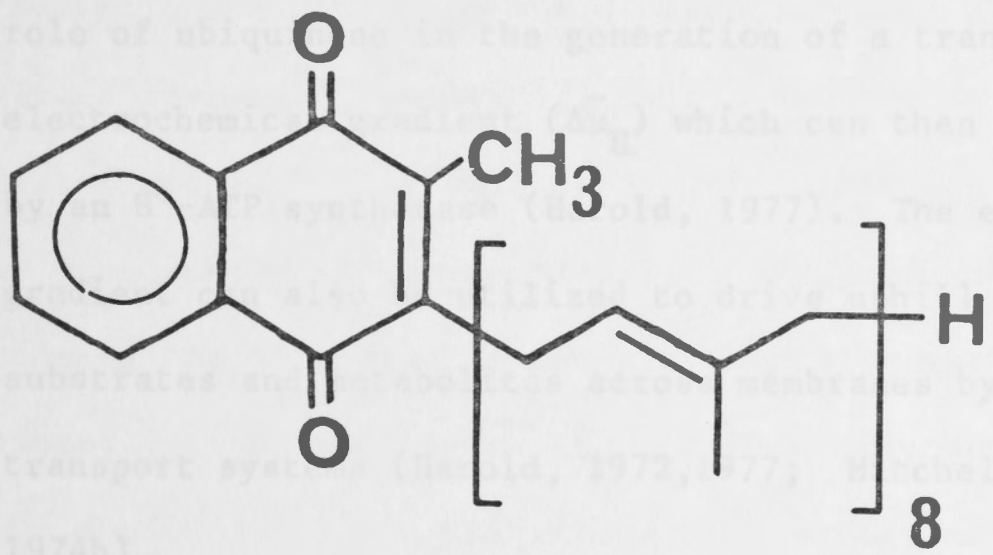
Two major structural groups of bacterial isoprenoid quinones can be recognized, the naphthoquinones and benzoquinones. Naphthoquinones can be divided further into two main types on the basis of structural considerations. These are the phylloquinones (associated with the green part of plants and occurs less commonly in bacteria) and the menaquinones (Collins and Jones, 1981). E. coli is one of a small group of organisms containing both ubiquinone and menaquinone (vitamin K_2) (Pandya and King, 1966), the structures of which are shown in Figure 1-2. The relative amounts of ubiquinone and menaquinone present in cells depends on the degree of aeration of the growth medium. Menaquinone has been found to be the major quinone in anaerobically grown cells whereas ubiquinone has been found to be present in amounts 20-fold that of menaquinone in cultures which have been vigorously aerated (Polglase, Pun and Withaar, 1966).

Extraction/reactivation studies in mitochrondria demonstrated that ubiquinone is an obligatory redox component of the mitochondrial electron transport chain (Lester and Fleischer, 1959; Ernster, Lee, Norling and Persson, 1969a,b). In the case of E. coli, the obligatory role of quinones in respiration has been verified by experiments involving mutants unable to form ubiquinone (ubi), menaquinone/demethylmenaquinone (men), or both quinones (ubi men)

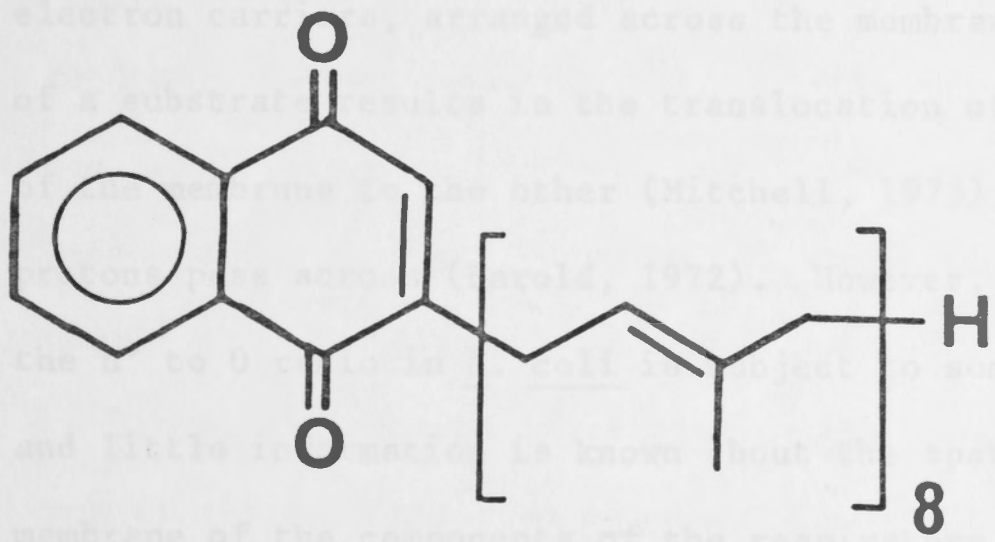
Figure 1-2. Structure of the Major Quinone Species in E. coli



Ubiquinone-8



Menaquinone-8



Demethylmenaquinone-8

(Cox, Newton, Gibson, Snoswell and Hamilton, 1970; Newton, Cox and Gibson, 1972; Gibson and Cox, 1973; Wallace and Young, 1977a,b). The role of ubiquinone in the E. coli respiratory chain is discussed further in the Introduction to Chapter 3.

Although the obligatory role of quinones in respiration is now accepted, the exact mechanism(s) and sites of quinone action are still in dispute. The development of the chemiosmotic theory of oxidative and photosynthetic phosphorylation, introduced by Mitchell (1961,1973) and considered by many authors to be experimentally proved (Skulachev, 1980), has greatly stimulated interest in the role of ubiquinone in the generation of a transmembranous electrochemical gradient ($\Delta\bar{\mu}_H$) which can then be utilized to form ATP by an H^+-ATP synthetase (Harold, 1977). The electrochemical proton gradient can also be utilized to drive uphill transport of many ions, substrates and metabolites across membranes by respiration-dependent transport systems (Harold, 1972,1977; Mitchell, 1973; Kaback, 1974b).

Central to the chemiosmotic hypothesis is the view that the respiratory chain is an alternating sequence of hydrogen carriers and electron carriers, arranged across the membrane in loops. Oxidation of a substrate results in the translocation of protons from one side of the membrane to the other (Mitchell, 1973); in any one loop, two protons pass across (Harold, 1972). However, the stoichiometry of the H^+ to O ratio in E. coli is subject to some dispute (Bragg, 1980) and little information is known about the spatial arrangement in the membrane of the components of the respiratory chain of E. coli.

Mitchell (1975a,b; 1976) has put forward a general formulation called the "protonmotive Q cycle" describing the role of ubiquinone in the hypothetical redox loops. Although the protonmotive Q cycle

hypothesis still awaits experimental verification it has stimulated interest in the biophysical behaviour of ubiquinones in both natural and artificial membrane systems.

There has been much debate about whether the quinones, which are well in excess of electron transfer chains, are a freely mobile species which form a homogeneous pool (Krȍger and Klingenberg, 1970; 1973a,b) or whether there are several protein bound forms which act as the carriers of reducing equivalents in the respiratory chain (Yu and Yu, 1981).

Ragan and Heron (1978) claimed that purified complex I and III from mitochondria, when mixed, associated in a 1:1 molar ratio to give a complex I-complex III unit. Furthermore, the ubiquinone associated with this unit does not equilibrate with other such units or free complex I and complex III. However, this stoichiometric 1:1 complex could only be formed at ubiquinone and phospholipid concentrations well below those within mitochondrial membranes. When the phospholipid and ubiquinone concentrations were raised, natural inner mitochondrial membrane behaviour was restored to the purified complex I-complex III system (ie the two associated complexes appeared to be linked by a mobile pool of ubiquinone molecules; Heron, Ragan and Trumpower, 1978). Heron *et al*. 1978 interpreted these results to mean that electron transfer occurs only through stoichiometric complex I-complex III units which are formed and reformed at rates higher than the rate of electron transfer.

Recent experiments investigating the lateral diffusion of integral electron transfer components have demonstrated a diffusion-limited step in electron transfer between the inner membrane dehydrogenases and cytochromes bc_1 and indicates that the

dehydrogenases, ubiquinone, and cytochromes bc_1 are independent, diffusible membrane oxidation-reduction components (Schneider, Lemasters, Höchli and Hackenbrock, 1980). Schneider *et al*. (1980) fused the inner membranes of mitochondria with exogenous soybean phospholipid. They demonstrated that electron transfer rates from NADH and succinate to cytochrome *c* as well as to oxygen decreased in proportion to the phospholipid enrichment and increased surface area of the membrane bilayer (examined and measured by freeze-fracture electron microscopy). In later experiments, the role of ubiquinone in this process was evaluated by fusing liposomes containing ubiquinone-10 or ubiquinone-6 with mitochondrial inner membranes (Schneider, Lemasters and Hackenbrock, 1982). In control membranes enriched with phospholipid only, ubiquinol-cytochrome *c* reductase and NADH- and succinate-cytochrome *c* reductase activities decreased proportionally to the increase in bilayer lipid. This effect was reversed by ubiquinone incorporation. The extent of recovery of NADH-, succinate-, and ubiquinol-cytochrome *c* reductase activity was related to the chain length of the newly incorporated ubiquinones with the shorter chain length, ubiquinone-6, giving greater recoveries than the longer chain length, ubiquinone-10. Since lateral mobility of a lipid molecule increases with decreasing chain length, the relationship between the chain length and activity also supports the view that ubiquinone mediates electron transfer by diffusion (Schneider *et al*., 1982). Also, these results obtained by Schneider *et al*. (1980) are not consistent with the model outlined by Heron *et al*. (1978), and Ragan and Heron (1978). Schneider *et al*. (1980) found that as exogenous phospholipid was incorporated into the membrane, NADH-cytochrome *c* reductase activity decreased much more

than did NADH-ubiquinone reductase activity or ubiquinol-cytochrome c reductase activity. A number of schematic models pertaining to electron transfer between the dehydrogenases and cytochromes bc_1 are presented in Figure 1-3.

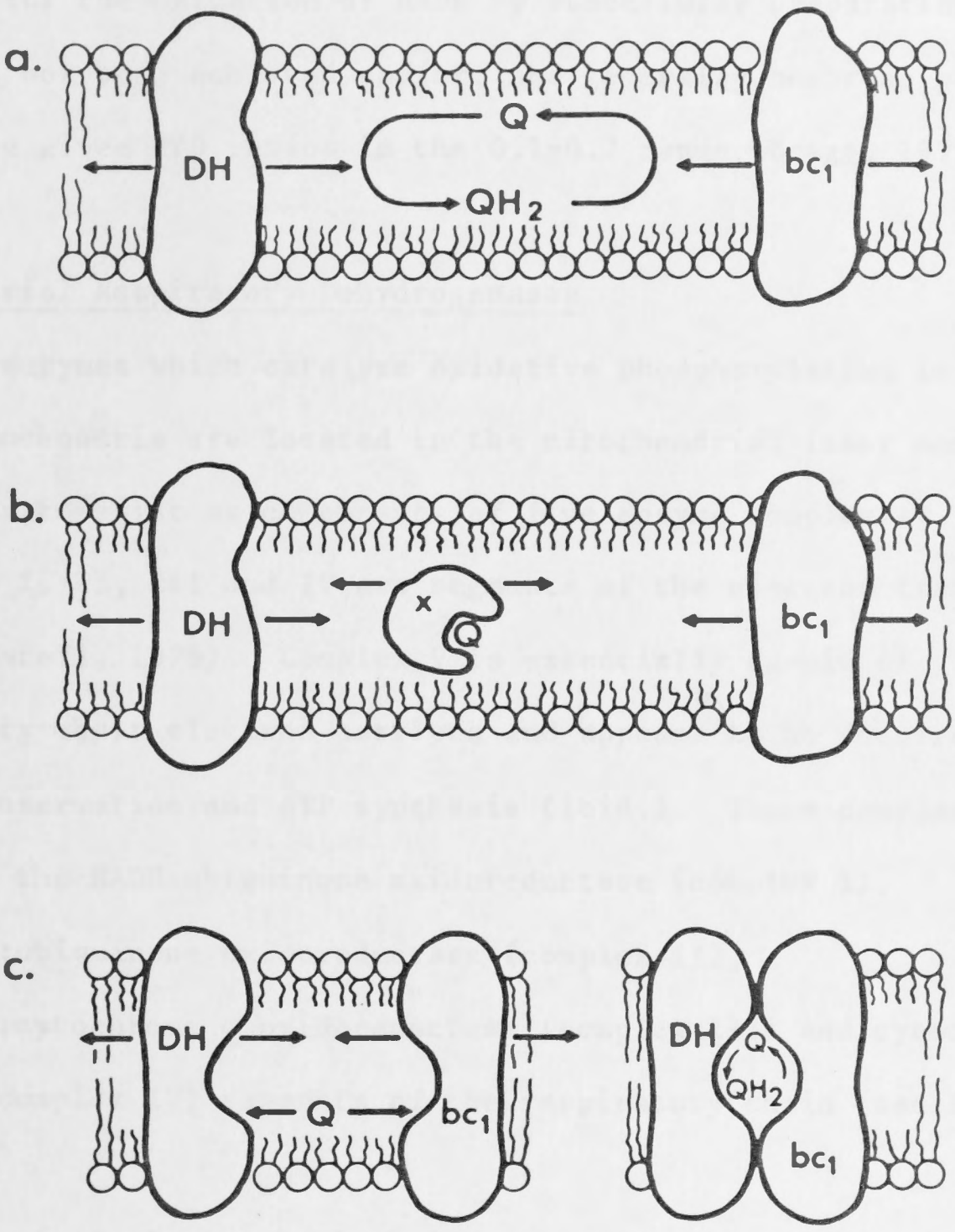
Sites of Proton Translocation and Energy Coupling

As pointed out by Hatefi (1976), the mechanisms of energy conservation and coupling in mitochondria are still obscure and the efforts of various groups in isolating coupling factors and reconstructing partial reactions of oxidative phosphorylation have not unravelled these central problems. Nonetheless, preparations of complex I are capable of energy coupling as shown by the reconstitution experiments of Ragan and Racker (1973) (referred to as 'site I'; sites of energy coupling are shown in Figure 1-4).

The nature and number of coupling sites in the E. coli respiratory chain have not been unambiguously determined. There have been several methods used to determine the efficiency of oxidative phosphorylation and the number of phosphorylation sites associated with the respiratory chain. One method which has been extensively used measures the stoichiometry of H^+ extrusion from cells in the presence of the permeant thiocyanate ion following the addition of a pulse of oxygen to an anaerobic cell suspension (see Bragg, 1979, for review). Several reports have indicated that there are two energy conservation sites associated with the respiratory chain of aerobically grown cells of E. coli (Lawford and Haddock, 1973; Farmer and Jones, 1976; Jones, Brice, Downs and Drozd, 1975).

If there are at least two coupling sites in the respiratory chain of E. coli, then a P/O ratio of 2 would be the upper value

Figure 1-3. Diffusional Models of Electron Transfer between Dehydrogenases and Cytochromes bc_1



(a) Dehydrogenases (DH), cytochrome bc_1 complex (bc_1), and ubiquinone (Q and QH_2) are independently diffusing electron transfer components.
(b) Same as (a), but ubiquinone (Q) is bound to a diffusible quinone binding protein (X) as a prosthetic group.
(c) Electron transfer through direct interaction between diffusible dehydrogenases (DH) and cytochrome bc_1 complex (bc_1) with ubiquinone (Q and QH_2) as a coenzyme. This figure is taken from Schneider et al., (1980).

obtained for the oxidation of NADH by subcellular preparations. This value has not been achieved so far, and generally membrane particles alone have given P/O ratios in the 0.1-0.2 range (Bragg, 1979).

Mitochondrial Respiratory Dehydrogenases

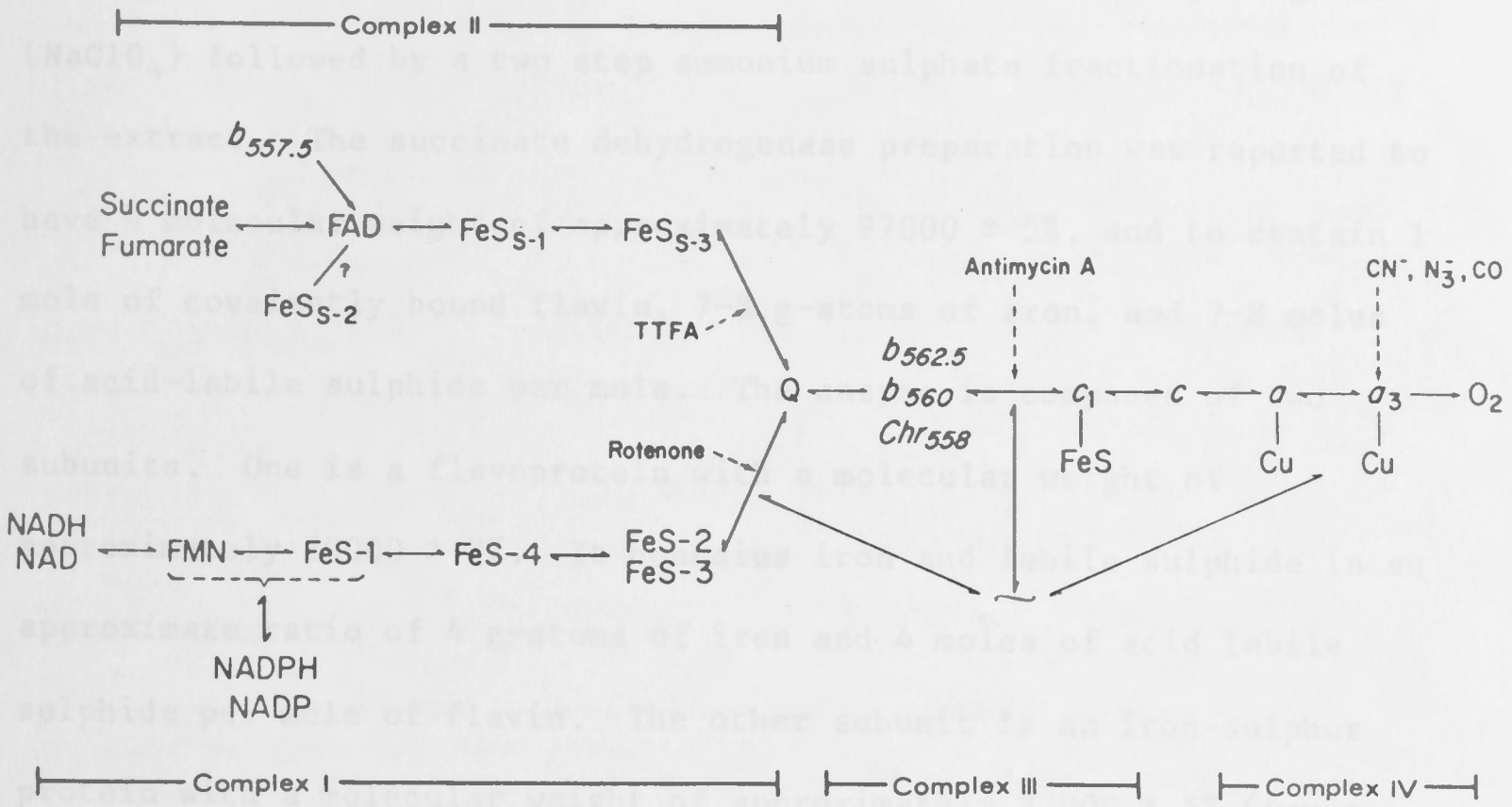
The enzymes which catalyze oxidative phosphorylation in bovine heart mitochondria are located in the mitochondrial inner membrane and appear to exist as components of five enzyme complexes. Complexes I, II, III and IV are segments of the electron transport system (Hatefi, 1976). Complex V is essentially devoid of respiratory-chain electron carriers and appears to be concerned with energy conservation and ATP synthesis (ibid.). These complexes represent the NADH:ubiquinone oxidoreductase (complex I), succinate:ubiquinone oxidoreductase (complex II), ubiquinol:cytochrome *c* oxidoreductase (complex III) and cytochrome *c* oxidase (complex IV) segments of the respiratory chain (see Figure 1-4).

The principal entry points into the mitochondrial respiratory chain, the respiratory NADH dehydrogenase and succinate dehydrogenase, have been studied intensively over the last twenty five years.

Complex II (Succinate:ubiquinone Oxidoreductase)

Succinate:ubiquinone oxidoreductase was first isolated by Ziegler and Doeg (1959). Preparations of complex II catalyze the oxidation of succinate by ubiquinone-2 or phenazine methosulphate (PMS) at a rate of 50 to 55 μmoles $min^{-1}mg^{-1}$ protein at $38^{o}C$ (Baginsky and Hatefi, 1969).

Figure 1-4. Respiratory Chain Components Present in the Inner Mitochondrial Membrane



Sequence of electron carriers in the respiratory chain showing the approximate sites for coupling at the level of complexes I, III, IV and the apparent inhibition sites for rotenone, 2-thenolytriluroacetone (TTFA), antimycin A, cyanide, azide and carbon monoxide. FeS, iron-sulphur centre. Figure is taken from Hatefi, (1976).

Davis and Hatefi (1971) purified a soluble succinate dehydrogenase from complex II of beef heart mitochondria by a simple procedure involving extraction in the presence of chaotropic agents ($NaClO_4$) followed by a two step ammonium sulphate fractionation of the extract. The succinate dehydrogenase preparation was reported to have a molecular weight of approximately 97000 ± 5%, and to contain 1 mole of covalently bound flavin, 7-8 g-atoms of iron, and 7-8 moles of acid-labile sulphide per mole. The enzyme is composed of two subunits. One is a flavoprotein with a molecular weight of approximately 70000 ± 7%. It contains iron and labile sulphide in an approximate ratio of 4 g-atoms of iron and 4 moles of acid labile sulphide per mole of flavin. The other subunit is an iron-sulphur protein with a molecular weight of approximately 27000 ± 5% (Davis and Hatefi, 1971). The same preparation was also shown to restore full activity to electron transport particles or complex II which had had its succinate dehydrogenase selectively destroyed at pH 9.3 (Hanstein, Davis, Ghalambor and Hatefi, 1971). The specific activity for this preparation is reported to be 67-78 μmoles succinate oxidized $min^{-1}mg^{-1}$ protein at 38°C using PMS as an acceptor (Hanstein et al., 1971). This is similar to the activity observed for preparations of complex II (Baginsky and Hatefi, 1969). The activity of the purified succinate dehydrogenase with ubiquinone-2 as acceptor has not been reported.

Interestingly, succinate dehydrogenases from several different sources have been purified and shown to possess very similar properties to the beef heart mitochondrial enzyme (see Table 1-2). Further characterization of these enzymes from diverse sources may provide some interesting data on the evolution of what appears to be a conserved enzyme system.

Table 1-2. Properties of the Respiratory Succinate Dehydrogenase Preparations Isolated from Various Sources

Organism	Flavoprotein M_r	Fe-S protein M_r	Reference
Beef heart mitochondria	70000±7%	27000±5%	Davis and Hatefi (1971)
Rhodospirillum rubrum	60000	25000	Hederstedt and Rutberg (1981)
Bacillus subtilis[a]	65000	28000	Hederstedt, Holmgren and Rutberg (1979)
Neurospora crassa[b]	72000	28000	Weiss and Kolb (1979)
Micrococcus luteus	72000	30000	Crowe and Owen (1983)

a this preparation also contains a 19000-dalton polypeptide.
b also contains a cytochrome b subunit (M_r probably 14000).

Complex I (NADH:ubiquinone Oxidoreductase)

In mammalian mitochondria, the respiratory NADH dehydrogenase (complex I) is believed to catalyze electron transfer from NADH to the endogenous ubiquinone-10. The primary dehydrogenase is not believed to catalyze electron transfer directly from NADH to ubiquinone-10 but a number of iron-sulphur proteins are thought to act as intermediate electron carriers (Hatefi and Stiggall, 1976; Ragan, 1976a; Beinert and Albracht, 1982).

Complex I contains 1 mole of FMN, 3 moles of ubiquinone-10, 16-18 g-atoms of iron, and 16-18 moles of acid-labile sulphide per 6.5-7 x 10^5 g protein (or 8-8.5 x 10^5 g protein plus lipid) (Hatefi, 1976).

Complex I catalyzes the reduction of ubiquinone-1 by NADH at a rate of 27.6 μmoles $min^{-1}mg^{-1}$ protein at 38°C (Hatefi, Haavik and Griffiths, 1962) with a K_m (ubiquinone-1 as acceptor) of 44 μM (Ragan, 1976a).

There has not been exact agreement on the number of polypeptides present in isolated preparations of complex I. One report claimed at least 17 peptides were present, whereas another suggested the presence of at least 26 polypeptides (Hatefi, Galante, Stiggall and Ragan, 1979; Ragan, 1976b; Heron, Smith and Ragan, 1979). Also, a number of polypeptides have been reported to occur in stoichiometric molar proportions (Ragan, 1976b). However, the number of these proteins actively involved in the oxidation of NADH by ubiquinone and the coupling of this process to the translocation of protons across the membrane is unknown.

The iron-sulphur protein isolated from complex I appears to be composed of three polypeptides with molecular weights of 75000, 53000

and 29000 (Ragan, 1976b). EPR spectrometry indicates that complex I contains four to six iron-sulphur clusters (Ohnishi, 1979; Beinert and Albracht, 1982), however, the exact sequence of electron transfer from NADH to ubiquinone via flavin and iron-sulphur centres is unresolved (see Figure 1-4).

Complex I has been successfully reconstituted into phospholipid vesicles by the cholate dialysis procedure (Ragan and Hinkle, 1975). Oxidation of NADH by ubiquinone-1 catalyzed by the reconstituted vesicles was coupled to proton translocation (ibid.).

Preparations of NADH dehydrogenase from mammalian mitochondria may be divided into three types: (a) NADH:ubiquinone oxidoreductase or complex I of the electron transport system, (b) the high molecular weight (type I) NADH dehydrogenase, and (c) the low molecular weight (type II) NADH dehydrogenase (Hatefi and Stiggall, 1976).

The type I NADH dehydrogenase (molecular weight ~800000) can be purified by snake venom phospholipase A digestion of complex I followed by $(NH_4)_2SO_4$ fractionation (Ringler, Minakami and Singer, 1963). Complex I and the type I NADH dehydrogenase, exemplified by the preparation of Ringler et al. (1963), differ primarily in that the latter preparation lacks phospholipid and endogenous ubiquinone and does not catalyze the rotenone-sensitive reduction of exogenous ubiquinone-1. However, the enzyme catalyzes the oxidation of NADH at a very high rate of 355 μmole $min^{-1}mg^{-1}$ protein at 30°C, with ferricyanide as acceptor (Ringler et al., 1963).

Degradation of the type I NADH dehydrogenase by a wide variety of treatments causes loss of the original activities and the emergence of new ones, in particular, rotenone-insensitive

NADH:ubiquinone oxidoreductase (Ragan, 1976a). This process is associated with the fragmentation of the dehydrogenase into a low-molecular-weight form (type II NADH dehydrogenase). This process can be achieved by treatment of the type I dehydrogenase with heat, acid and ethanol (Watari, Kearney and Singer, 1963) or with heat alone, with proteolytic enzymes or with urea (Cremona, Kearney, Villavicencio and Singer, 1963). It was found by Singer and his colleagues that the products isolated after the above mentioned treatments were surprisingly similar (Cremona et al., 1963).

A type II NADH dehydrogenase has been isolated in purified, soluble form after resolution of complex I by chaotropic agents (Hatefi and Stempel, 1969; Hatefi, Stempel and Hanstein, 1969). The enzyme preparation is water soluble and has a molecular weight of 69000 ± 1000 (Galante and Hatefi, 1979). It is an iron-sulphur flavoprotein composed of three subunits with apparent M_r values of 51000, 24000 and 9-10000. The amino acid compositions of each of the isolated and purified subunits has been determined (ibid.). This preparation, in contrast to the isolated type I dehydrogenase which lacks ubiquinone-1 reductase activity, catalyzes the reduction of ubiquinone-1 by NADH at high rates (175 μmole NADH oxidized $min^{-1}mg^{-1}$ protein at 38°C; Hatefi and Stempel, 1969). However, unlike the ubiquinone-1 reductase activity of complex I, the type II dehydrogenase ubiquinone-1 reductase activity is not as readily inhibited by rotenone, piericidin or seconal (Hatefi et al., 1969). Also, although the ubiquinone-1 reductase activity of complex I is inhibited by guanidine, the ubiquinone-1 reductase activity of the type II NADH dehydrogenase preparation is activated by guanidine (ibid.).

Consequently, the high ubiquinone reductase activity of the type II dehydrogenase preparation has been considered by some authors to be largely artifactual (for discussion see: Singer and Gutman, 1971; Ragan, 1976a; Hatefi and Stiggall, 1976).

The controversies in the literature concerning the complex I-derived NADH dehydrogenases (types I and II) illustrate the difficulties that can be encountered in the biochemical characterization of membrane-bound enzyme complexes. Recombinant DNA methods have yet to be applied to the study of the mitochondrial respiratory complexes.

The recombinant DNA approach is more readily applied to those mitochondrial proteins encoded in the mitochondrial genome and the recent determination of the complete DNA sequence of the human and bovine mitochondrial genomes has provided a great deal of new information concerning this interesting energy transducing system (Anderson, Bankier, Barrell, de Bruijn, Coulson, Drouin, Eperon, Nierlich, Roe, Sanger, Schreier, Smith, Staden and Young, 1981; Anderson, de Bruijn, Coulson, Eperon, Sanger and Young, 1982). These methods have great potential and have been successfully utilized in some studies of the E. coli respiratory chain where the availability of mutants provides significant advantages.

Bacterial Membrane-Bound NADH Dehydrogenases

A number of bacterial membrane-bound NADH dehydrogenases have been purified (Table 1-3). Only membrane-bound enzymes that are specific for NADH oxidation and are capable of reducing ubiquinones or artificial electron acceptors are included in the table. Often the purities of the various preparations were not given.

Table 1-3. Properties of Bacterial Membrane-Bound NADH Dehydrogenases

Organism	Apparent Mol Wt	Prosthetic Group	Specific Activity	Acceptor	Reference
Escherichia coli	47000	FAD	500-600[a]	Ubiquinone-1	Jaworowski, Campbell, Poulis and Young (1981a); Jaworowski, Mayo, Shaw, Campbell and Young (1981b)
Acholeplasma laidlawii[b]	nd[c]	nd	23.2[b]	$Fe(CN)_6^{3-}$	Jinks and Matz (1976)
Halophilic bacterium AR-1	64000	FAD	376[d]	DCIP[e]	Hockstein and Dalton (1973)
Photobacterium phosphoreum (marine bacterium)	nd	FAD	24.2[f]	$Fe(CN)_6^{3-}$	Imagawa and Nakamura (1978)
Bacillus stearothermophilus (thermophilic bacterium)	43000	FMN	94.0[g]	DCIP	Mains, Power, Thomas and Buswell (1980)
Peptostreptococcus elsdenii	Two nonidentical subunits of 41000 and 33000	FAD[i]	19.6[j]	DCIP	Whitfield and Mayhew (1974)
Micrococcus lysodeikticus	140000 (dimer of 70000)	FAD	nd	DCIP	Zhukova, Kharat'yan and Ostrovskii (1979)
Bacillus caldotenax (thermophilic bacterium	44000	FAD	243[a]	DCIP	Kawada, Takeda and Nosoh (1981)
Bacillus subtilis	63000	FAD	24.7[k]	3-(4',5'-dimethyl -thiazol-2-yl) -2,4-diphenyl tetrazolium bromide	Bergsma, Van Dongen and Konings (1982)

Table 1-3. (continued)

Organism	Apparent Mol Wt	Prosthetic Group	Specific Activity	Acceptor	Reference
Acinetobacter calcoaceticus	34000	FMN	0.43[d]	DCIP	Borneleit and Kleber (1983)
Alkalophilic Bacillus	Two identical subunits of 65000	FAD	74.3[d]	DCIP	Hisae, Aizawa, Koyama, Sekiguchi and Nosoh (1983)
Rhodospirillum rubrum (phototrophic bacterium)	26000	FMN	49.8	DCIP[1]	Horio, Bartsch, Kakuno and Kamen (1969)
Rhodopseudomonas capsulata (phototrophic bacterium)	97000±5000 (composed of six identical subunits, 15500 each)	nd	15.4	DCIP	Ohshima and Drews (1981)

a μmoles NADH oxidized min^{-1} mg^{-1} protein at 30°C.
b Organism contains no quinones or cytochromes.
c nd = not determined.
d μmoles DCIP reduced min^{-1} mg^{-1} protein at 30°C.
e DCIP = 2,6-dichlorophenolindophenol.
f μmoles $Fe(CN)_6^{3-}$ reduced min^{-1} mg^{-1} at 25°C.
g Same as for d but at 55°C.
h Same as for a but at 37°C.
i This unusual enzyme preparation has 1 FAD associated with each subunit. However, the isolated protein contains various amounts of modified flavins.
j Specific activity is defined as absorbance change of 1 min^{-1} mg^{-1} protein at A_{600} and 25°C.
k μmoles 3-(4'-5'-dimethyl-thiazol-2-yl) 2,4-diphenyltetrazolium bromide reduced min^{-1} mg^{-1} protein at 37°C.
l μmoles DCIP reduced min^{-1} A_{280}^{-1}.

According to a major review of the distribution of isoprenoid quinone structural types in bacterial taxa (Collins and Jones, 1981) most of the organisms listed in Table 1-3 possess either menaquinone and/or ubiquinone. The exceptions are A. laidlawii which does not possess quinones and P. elsdenii for which information is lacking. One uncertainty about the enzymes listed in Table 1-3 (except for the E. coli enzyme) is that although they are all membrane bound NADH dehydrogenases, the activities of the enzyme preparations towards their physiological quinone acceptor have not been determined. Furthermore, no other evidence is provided to prove their direct involvement in respiration.

Some of the enzymes presented in Table 1-3 differ markedly in their subunit molecular weights which would be likely to preclude any close evolutionary relatedness (eg M_r 47000 for the E. coli enzyme versus M_r 15500 for the R. capsulata enzyme). This is not unexpected since a number of the enzymes in Table 1-3 have been purified from widely different bacterial taxa. Nonetheless, the fact that the respiratory succinate dehydrogenase isolated from such phylogenetically distant sources as beef heart mitochondria, N. crassa mitochondria, R. rubrum, B. subtilis and M. luteus show similar subunit compositions would suggest that at least some components of the respiratory chain have been conserved in their evolution.

Eleven of the respiratory-chain-linked NADH dehydrogenases listed in Table 1-3 have been shown to contain or at least be activated by flavin (FAD or FMN). It will be interesting to observe, when sequence data becomes available, whether these flavoproteins possess a conserved flavin binding domain as has been shown for the

fumarate reductase of E. coli and the succinate dehydrogenase from beef heart mitochondria (Cole, 1982).

The Respiratory NADH Dehydrogenase of E. coli

There have been a number of attempts to purify the respiratory NADH dehydrogenase of E. coli. Early reports of purification attempts includes those of Wosilait and Nason (1954), Bragg (1965), Bragg and Hou (1967a,b), and Gutman, Schejter and Avi-dor (1968). In these early reports the purity of the various preparations were not ascertained. Furthermore, the activities of the preparations with natural quinones, rather than artificial electron acceptors, were not cited. Also, frequently the isolated preparations differed markedly in their response to inhibitors from the NADH dehydrogenase of intact membrane vesicles.

The NADH dehydrogenase of the E. coli respiratory chain has been identified (Dancey, Levine and Shapiro, 1976) by the following properties: (a) its location in membrane vesicles, (b) its inhibition by AMP in a fashion similar to that of the NADH oxidase, (c) its specificity for NADH, but not NADPH, with the same K_m for NADH as that of the NADH oxidase (50 μM and 33 μM, respectively), and (d) its sensitivity to inhibition by HQNO, dicoumarol and rotenone which are also inhibitors of the NADH oxidase. The NADH dehydrogenase of the cytosol fraction (assayed as NADH:DCIP oxidoreductase activity) differs substantially from the membrane-bound activity both in substrate specificity and in the inhibitors of the reaction (Dancey et. al., 1976).

Dancey, Levine and Shapiro (1976) reported the isolation of the respiratory NADH dehydrogenase by solubilization of membrane vesicles

with Triton X-100 followed by chromatography on DEAE-cellulose, precipitation by 30% ethanol, and chromatography on hydroxylapatite and DEAE-agarose. The preparation was shown to catalyze the reduction of $Fe(CN)_6^{3-}$, DCIP and menadione. However, activity with natural ubiquinone homologues was not reported. Also, the specific activity with DCIP as electron acceptor was very low (0.32 µmole DCIP reduced $min^{-1}mg^{-1}$ protein). It is noteworthy that although HQNO (at 10 µM concentration) inhibits the respiratory NADH dehydrogenase it is ineffective as an inhibitor of the NADH:DCIP oxidoreductase isolated by Dancey et. al. (1976).

Electrophoresis on SDS-polyacrylamide gels showed one band of mol wt 38000 which in the best preparations accounted for 75% of the protein on the gel (ibid.).

Dancey and Shapiro (1976) characterized the 38000 mol wt preparation further and demonstrated that the enzyme contains no flavin but has an absolute requirement for added flavin for activity. An antibody preparation elicited against the purified enzyme was demonstrated to be effective as an inhibitor of the membrane-bound NADH dehydrogenase and the NADH oxidase activities (ibid.). This antibody preparation produced two major precipitates when used to examine solubilized membrane preparations.

Thompson and Shapiro (1981) purified an NADH:ubiquinone oxidoreductase from Triton X-100 solubilized E. coli membranes. The enzyme was purified 100-fold after solubilization by chromatography on AMP-Sepharose, DEAE-Sephacel and Bio-GelA-1.5M in Triton X-100 containing buffers. The described NADH:ubiquinone oxidoreductase has an M_r of 46000 as determined by SDS-polyacrylamide gel electrophoresis. However, a protein of M_r 37000 was present in every preparation and sometimes appeared as a doublet.

Thompson and Shapiro (1981) concluded from atomic absorption spectroscopy data that there is about 1 mole of Fe/46000 daltons of protein. However, only small amounts of quinone reductase were used in these measurements (5 μg mL^{-1}).

The purification protocol developed by Thompson and Shapiro (1981) gave a low yield of 0.32 mg purified enzyme from 300 g wet weight of cells. Nonetheless, this enzyme preparation was active with ubiquinone-3, DCIP and $Fe(CN)_6^{3-}$ as electron acceptors. The specific activity with ubiquinone-3 as acceptor was 50 μmole of NADH oxidized $min^{-1}mg^{-1}$ protein with a calculated apparent K_m of 40 μM.

Thompson and Shapiro (1981) showed that antiserum prepared against the purified NADH:DCIP oxidoreductase (Dancey and Shapiro, 1976) inhibits the NADH oxidase activity of the intact membranes as well as the purified NADH:ubiquinone oxidoreductase. Furthermore, the antiserum immunoprecipitated both the M_r 46000 polypeptide and the M_r 37000 polypeptide that is present in all preparations at 5-30% of the amount of M_r 46000. As pointed out by Thompson and Shapiro (1981), one explanation for the antigenic cross-reactivity of the M_r 46000 and the M_r 37000 polypeptides is that the smaller may be a proteolytic cleavage product of the larger. However, although the data was not shown, Thompson and Shapiro (1981) stated that the two polypeptides produce distinct peptide patterns when digested with either Staphylococcus aureus V8 protease or trypsin. They concluded that it is unlikely that one protein is a cleavage product of the other.

Young and Wallace (1976) isolated a strain of E. coli carrying a point mutation affecting the respiratory NADH dehydrogenase complex. The evidence for the genetic lesion in this strain (AN589; Hfr; metB

ndh-401) being in the respiratory NADH dehydrogenase complex includes: (a) electron transport to oxygen from succinate and D-lactate is not impaired whereas the NADH oxidase is <2% of wild type levels, (b) comparison of the quinone levels of AN589 and a wild-type strain indicate that the ndh strain is not affected in the synthesis of ubiquinone or menaquinone, and (c) the kinetics of reduction of cytochrome b with NADH as substrate are consistent with the lesion in the electron transport system of the ndh strain being prior to cytochrome b.

The phenotype used for scoring strains lacking NADH oxidase in genetic experiments is growth on glucose plus succinate but not on mannitol as sole carbon source. The inability to grow with mannitol as sole carbon source may be because the conversion of mannitol-1-phosphate to fructose-6-phosphate generates one molecule of NADH which is not reoxidized in the subsequent metabolism of fructose to lactate and which is presumably oxidized via NADH oxidase in the wild type cell (Young and Wallace, 1976).

The gene coding for the respiratory NADH dehydrogenase of E. coli has been cloned (Young, Jaworowski and Poulis, 1978). Young et al. (1978) used the restriction endonuclease EcoRI to cleave a preparation of the E. coli chromosomal DNA and the fragments produced were ligated into EcoRI digested pSF2124 (So, Gill and Falkow, 1975). The ligated hybrid plasmids were transformed into an F^+ derivative of strain C600. Transformed cells were selected by growth in medium containing ampicillin. These cells were grown in the presence of the recipient IY12 (F^-, ndh rpsL) and the hybrid plasmids were transferred by F-mobilization (Young et al., 1978). The cells were then selected for the complementation of ndh (growth on mannitol;

Young and Wallace, 1976). Young et al. (1978) isolated a strain (IY35) which possesses the hybrid plasmid pIY1 carrying the ndh gene. Electrophoresis of the EcoRI digested pIY1 on an agarose gel indicated that it carries a 1.6 Mdal (~2.5 kb) DNA fragment which possesses the ndh gene. Cells containing the plasmid are found to possess 8-10 times the normal level of NADH:ubiquinone oxidoreductase in their membranes. Moreover, when the cells were treated with chloramphenicol to increase the plasmid copy number, the level of NADH dehydrogenase in the membrane can be increased to 50-60 times the level in the wild type (Young et al., 1978).

The aforementioned genetic amplification method has been successfully utilized in the purification protocol of the respiratory NADH dehydrogenase of E. coli reported by Jaworowski, Campbell, Poulis and Young (1981a). However, as well as the IY35 strain a newly constructed strain, IY85, was also described. IY85 carries the plasmid pIY9, which is derived from the vector pSF2124 and carries a double lac promoter inserted adjacent to the ndh fragment of pIY1 (Jaworowski et al., 1981a).

The NADH:ubiquinone oxidoreductase was purified to homogeneity by hydroxylapatite chromatography following potassium cholate solubilization of membrane vesicles. The use of multicopy plasmid vectors carrying the ndh gene facilitated the genetic amplification of strains which enabled the enzyme to be purified 800- to 1000-fold relative to the activity in wild-type membranes. The use of a large scale purification procedure enables 50-100 mg of protein with a specific activity of 500-600 μmole of NADH oxidized $min^{-1}mg^{-1}$ protein with ubiquinone-1 as electron acceptor to be obtained. SDS-polyacrylamide gel electrophoresis demonstrated that the purified

enzyme consists of a single polypeptide with a reported M_r of 47000 (Jaworowski *et al.*, 1981a,b).

The purification protocol developed by Jaworowski *et al.* (1981a) produces a similar preparation to that reported by Thompson and Shapiro (1981), although it gives considerably greater quantities of enzyme. The purified NADH:ubiquinone oxidoreductase has been further characterized by Jaworowski, Mayo, Shaw, Campbell and Young (1981b). They showed that the enzyme preparation contains 70% (w/w) lipid, predominantly phosphatidylethanolamine and one mole of noncovalently bound FAD/mole of enzyme subunit. Interestingly, Jaworowski *et al.* (1981b) reported that the concentrated pure enzyme had 0.13 mole of Fe/mole of enzyme subunit, contradicting the 1:1 stoichiometry cited by Thompson and Shapiro (1981). More recently Campbell (unpublished) examined the metal content of the pure enzyme and demonstrated by neutron activation analysis the absence of 35 trace elements including Fe, Se, Zn, Mn, Co, W and Cu.

The purified enzyme has also been demonstrated to reconstitute NADH oxidase activity when added to membrane vesicles prepared from *ndh* strains (Jaworowski *et al.*, 1981b).

The genetic methods utilized by Young *et al.* (1978) and Jaworowski *et al.* (1981a) offer several important advantages over the more traditional biochemical approaches used by their predecessors. Firstly, the genetic cloning and amplification of a gene encoding a membrane protein, present as a minor component of the inner membrane, allows high-yield purification methods to be devised. Secondly, the cloning of the gene provides the opportunity for the rapid determination of the DNA sequence. This is in contrast to traditional protein sequencing methods which can encounter

difficulties with regards to membrane proteins. Thirdly, the sequence of the structural gene can potentially lead to the discovery of hitherto unknown genetic mechanisms (for example, the extreme economy of the genes and atypical codon usage in the human mitochondrial genome; Anderson et al., 1981).

Research Presented in this Thesis

The work described in this thesis is part of a multidisciplinary approach to the isolation and molecular characterization of the respiratory NADH dehydrogenase of E. coli. Chapter 2 describes the determination and analysis of the DNA sequence of the structural gene coding for this enzyme. This work led to the identification of UUG as an initiation codon. The DNA sequencing work also yielded the complete amino acid sequence of the protein and showed that no signal peptide was present. Comparative sequence analysis enabled two nucleotide binding sites to be identified and demonstrated significant homology between the E. coli enzyme and human glutathione reductase. Detailed comparisons with the latter enzyme suggested similar domain structure for the FAD and NADH domains of the NADH dehydrogenase and human glutathione reductase. The remainder of the thesis concerns different aspects of the membrane biology of the NADH dehydrogenase. This difficult area represents the major gap in the characterization of this enzyme. Most of the approaches were carried out with the ultimate goal of correlating the observed biological properties with features of the amino acid sequence. In Chapter 3, evidence is provided to demonstrate that the membrane bound respiratory coenzyme ubiquinone-8 is the physiological electron acceptor for the NADH dehydrogenase in vivo. Chapter 4 describes

experiments on the reconstitution of the NADH dehydrogenase into natural and artificial membranes and also attempts to define the membrane sector(s) of the enzyme by limited proteolysis. The use of photoaffinity labelling to study the ubiquinone binding site is described in Chapter 5. In Chapter 6 the partial DNA sequence for the respiratory D-lactate dehydrogenase of *E. coli* is described. This membrane bound enzyme also appears to use ubiquinone-8 as an electron acceptor *in vivo*. Although time did not permit the completion of this sequence it is anticipated that future comparison of the sequences of the two enzymes could yield interesting information concerning membrane interactions and ubiquinone catalysis.

Chapter 2

Nucleotide Sequence Coding for the *Ndh* Gene

INTRODUCTION

Rapid DNA Sequencing Methods

Over the last five years there has been dramatic progress in the development of methods used to determine primary nucleotide sequences (for reviews see: Wu, 1978; Sanger, 1981; Gilbert, 1981; Davies, 1982). This introduction confines itself to a discussion of the chain termination method since this method is used in the work described in this Chapter.

Sanger, Nicklen and Coulson (1977), in a pioneering work, described a method of DNA sequencing with chain-terminating inhibitors. The principle of this method is briefly described below. A primer and template are incubated with DNA polymerase in the presence of a mixture of deoxynucleoside triphosphates (dATP, dGTP, dCTP and dTTP), one of which has a radioactive [α-^{32}P] phosphate, allowing the newly synthesized DNA to be labelled. In each of the four dNTP reaction mixes a 2',3'-dideoxynucleoside triphosphate (ddATP, ddGTP, ddCTP or ddTTP) is also added. Because the ddNTPs contain no 3'-hydroxyl group, the chain cannot be extended further, so that termination occurs at positions where the ddNTPs are incorporated. The relative concentrations of dNTPs and ddNTPs are adjusted so that only a partial incorporation of the dideoxy analogue occurs at each possible site. Thus, by adding one of the dideoxy analogues to each of four separate incubations, four different groups of fragments ending at a particular base are produced. The other end of all fragments is identical, so that parallel size fractionation of the denatured fragments by analytical polyacrylamide gel electrophoresis allows the sequence to be deduced. Sanger *et al*.

(1977) also described the use of another chain-terminating analogue, the arabinonucleosides. Subsequently, this analogue has not been as extensively used as the dideoxynucleotides and hence it will not be discussed further.

In the publications by Sanger et al. (1977) and Sanger and Coulson (1978) the primers used in the sequencing reactions were purified restriction fragments from the double stranded replicative form of ØX174. The template used was single-stranded ØX174 DNA (ibid.). Since most DNA's are not readily available in a single-stranded form this approach was not widely applicable and methods such as the exonuclease method of Smith (1979) were developed to generate single stranded templates.

A major advance in sequencing methodology came as a direct result of the construction of an M13 cloning vehicle (designated M13mp1) by Messing, Gronenborn, Müller-Hill and Hofschneider (1977). Messing et al. (1977) inserted into a nonessential region of M13 a HindII restriction fragment comprising the E. coli lac regulatory region and the genetic information for the α peptide of β-galactosidase. Gronenborn and Messing (1978) modified the previously described M13mp1 by introducing by mutagenesis a unique EcoRI site into the lac region (the new derivative was designated M13mp2). They also confirmed that insertion of EcoRI fragments into M13mp2 leads to the inactivation of the α-complementation by the vector.

The use of the M13 derivatives described by Messing et al. (1977) and Gronenborn and Messing (1978) offered several major advantages over other vectors of that time. These advantages include: (a) upon infection, phage DNA is converted into a double stranded supercoiled replicative form (RF) and amplified to about

300 copies per cell, and the phage-producing cells do not lyze but continue to replicate, (b) because bacterial growth is slightly retarded by infection, infected bacteria can be detected by 'turbid-plaque' formation and no selective pressure (eg drug resistance factors) would be necessary to detect bacteria transfected by M13 DNA, and (c) insertion of DNA fragments at the unique EcoRI site results in a decrease in α-complementation allowing the identification of recombinant stages in an appropriate host on indicator plates (Messing et al., 1977; Gronenborn and Messing, 1978).

The single stranded M13mp2 proved to be suitable for use as a template in the Sanger chain-terminating sequence procedure (Schreier and Cortese, 1979; Sanger, Coulson, Barrell, Smith and Roe, 1980; Heidecker, Messing and Gronenborn, 1980). Sanger et al. (1980) and Heidecker et al. (1980) produced a major change in strategy in that the same primer is used on different templates, whereas in the previously reported dideoxy method (Sanger et al., 1977) different primers were used on one template. This approach is based on the cloning of small fragments of DNA into M13mp2 using a linker oligonucleotide. A specific primer which hybridizes close to the insertion site is used to determine the nucleotide sequence of each inserted fragment (Sanger et al., 1980).

A number of specific primers have been described which hybridize to the β-galactosidase gene flanking the EcoRI site of M13mp2. Schreier and Cortese (1979) described the use of a 96 bp EcoRI restriction fragment which hybridized to the DNA sequence corresponding to amino acid 7 to 35 of the β-galactosidase gene. Heidecker et al. (1980) described the use of a similar primer

(EcoRI/AluI restriction fragment) which extended from bp 13 to 106 of the lacZ gene. However, the disadvantage of both of these primers is their excessive length, approximately four times longer than is necessary for a stable specific interaction with the template. Hence, a shorter primer, a 26 bp EcoRI/BamHI fragment was synthesized and cloned (Anderson, Gait, Mayol and Young, 1980).

The development of methods for the rapid synthesis of oligodeoxyribonucleotides has enabled specific, single-stranded, heptadecamers to be synthesized and used as primers in the M13mp2 (or its derivatives) DNA sequencing system (Duckworth, Gait, Goelet, Hong, Singh and Titmas, 1981). Subsequently, several oligodeoxyribonucleotide primers have become commercially available and they will not be discussed further here. The ability to synthesize specific single stranded primers should, in theory, allow DNA sequencing of fragments cloned into normal plasmid vectors and several reports have been published recently demonstrating the validity of this approach (Wallace, Johnson, Suggs, Miyoshi, Bhatt and Itakura, 1981; Vieira and Messing, 1982).

A series of different derivatives of M13mp2 (Gronenborn and Messing, 1978) have been produced by the introduction of new cloning sites by the insertion of synthetic DNA into the β-galactosidase gene. The most notable derivatives include: M13mp5 (Messing, 1979), M13mp7 (Messing, Crea and Seeburg, 1981), and M13mp8 and M13mp9 (Messing and Vieira, 1982).

The development of the M13mp2 derivatives with a multipurpose cloning site has led to the use of the 'shotgun' system of DNA sequencing (Messing et al., 1981). Hence, large quantities of DNA can be cleaved with an assortment of restriction endonucleases

(single or double digests) and the DNA cloned into M13mp7, 8 or 9. Sanger and his coworkers, in a major achievement, used an assortment of methods, including the 'shotgun' system, to determine the complete nucleotide sequence of bacteriophage λ (48502 bp; Sanger, Coulson, Hong, Hill and Petersen, 1982).

Essential to the execution of the 'shotgun' random sequencing method is the application of computer programs suited to handle DNA sequence data. Numerous computer programs have been reported which have been specifically designed for storage, editing and analysis of both DNA and amino acid sequences (see Nucleic Acids Research Vol 10). In this laboratory the 'DNA programs' written by Roger Staden have been used (PDP FORTRAN PDP 11/45, Staden, 1977;1978;1979;1980; programs in FORTRAN 77 (VAX 11/780), Staden, 1982).

This Chapter describes the determination of the nucleotide sequence of the gene coding for the respiratory NADH dehydrogenase of E. coli by the chain termination method.

MATERIALS AND METHODS

All chemicals used were of the highest purity commercially available.

5-Bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-gal) was stored at 20°C as a 40 mg mL^{-1} stock solution in demethylformamide.

Deoxythymidine-5'-triphosphate, sodium salt (and other deoxynucleotides, dNTPs) were obtained from Boehringer Mannheim GmbH, and stored at -20°C as 20 mM stock solutions in 10 mM Tris

HCl, 0.1 mM EDTA, pH 7.5 buffer ($T_{10}E_{0.1}$, pH 7.5). 2',3'-Dideoxythymidine-5'-triphosphate, sodium salt (and other dideoxynucleotides, ddNTPs) were obtained from P-L Biochemicals (Milwaukee, Wis).

Restriction endonucleases were supplied by New England, Biolabs, Inc. DNA polymerase (enzyme A according to Klenow) was obtained from Bohringer Mannheim GmbH (Klenow, Overgaard-Hansen and Patkar, 1971).

Deoxyadenosine-5'-[α-^{32}P] triphosphate, triethylammonium salt ([α-^{32}P]dATP) was provided by The Radiochemical Centre, Amersham, England. The specific activity of the [α-^{32}P]dATP was 2000-3000 Ci/m mole (~74-111 TBq/m mole) and its radioactive concentration was 1 mCi/mL (37 MBq/mL).

Bacterial Strains and Plasmids

Young, Jaworowski and Poulis (1978) described the construction of a plasmid, pIY1, which contains a 1.6 Mdal (~2.5 kb) fragment inserted into the *Eco*RI site of the cloning vector pSF2124 (So, Gill and Falkow, 1975). The 1.6 Mdal insert contained the *ndh* structural gene which was obtained from an *Eco*RI digest of the chromosomal DNA of the *E. coli* K12 strain AN595 (*thi his ilv trp rpsL*) (Young and Wallace, 1976). *E. coli* K12 71-18 (Δ[*lac, pro*], F'*lac* $I^qZ\Delta M15$ *pro*$^+$) was used as a host for M13 (Messing *et al.*, 1977; Gronenborn and Messing, 1978). The single stranded phage vectors M13mp2 (Gronenborn and Messing, 1978; Sanger *et al.*, 1980) and M13mWJ22 (Rothstein, Lall, Bahl, Narang and Wu, 1980) were used as cloning vectors.

Strain IY35, derived from IY12 (*thi his ilv trp rpsL ndh*) (Young and Wallace, 1976), which possesses the hybrid plasmid pIY1 carrying *ndh* was used for the isolation of plasmid DNA. For the isolation

of plasmid DNA 1 L cultures were grown in glucose-minimal salts medium (Stroobant, Young and Gibson, 1972) containing 0.5% (w/v) casamino acids and the plasmid DNA was amplified using chloramphenicol (Clewell, 1972). The cells were harvested and a cleared lysate prepared according to the method of Clewell and Helinski (1969) except that 0.3% w/v Triton X-100 was used instead of brij and deoxycholate. The DNA was purified using a CsCl-ethidium bromide density gradient (Clewell, 1972), the ethidium bromide removed by isopropanol extraction and the DNA dialyzed against (2 mM Tris HCl, 2 mM EDTA, 1 mM NaCl, pH 8.0).

Transfection of Strain 71-18 with M13mp2 DNA

Strain 71-18 cells were grown at 37°C in 20 mL YT medium (5 g yeast extract, 8 g tryptone, 5 g NaCl in 1 L) to a Klett reading of 140 (Klett-Summerson colorimeter). The cells were harvested by low speed bench centrifugation for 5 minutes. The cells were resuspended in the same volume of 50 mM $CaCl_2$ (prechilled on ice) and mixed gently. The cells were pelleted and resuspended in 10 mL of 50 mM $CaCl_2$, mixed gently and held on ice for 15 minutes. The cells were pelleted and then resuspended to 1 mL volume in 50 mM $CaCl_2$. Transfection DNA (eg ligation mixture), about 10 ng per 200 µL of cell suspension, was added to the cell mix. The cells were held on ice for 30 minutes. The cells were then placed at 42°C for 3 minutes and then added to a soft agar overlay preparation containing isopropyl-β-D-thio-galactopyranoside (IPTG) and X-gal.

Titration of M13mp2: Detection of Recombinant Plaques

3 mL of 0.7% agar in YT medium was autoclaved and cooled to 42°C. 20 µL of X-gal stock solution (40 mg mL^{-1} in

demethylformamide) and 20 µL of IPTG stock solution (20 mg mL^{-1}) were added. The agar was mixed and then 200 µL of M13mp2 transfected 71-18 cells was added and mixed and the soft agar quickly poured onto a pre-warmed (37°C) YT plate. The plates were incubated for 24 hours at 37°C.

Strain 71-18, in which the entire lac operon is deleted, harbours an F episome which carries a deletion in the operator proximal region of the β-galactosidase gene (Messing et al., 1977; Gronenborn and Messing, 1978). M13mp2 has a HindII insert which codes for the lac regulatory region and the base sequence for the first 145 amino acids of the β-galactosidase (ibid.). It has been shown that the peptide produced by this fragment complements the episome encoded protein and consequently, strain 71-18 cells that are infected with M13mp2 can be detected by their blue colour on IPTG/X-gal indicator plates (ibid.). Furthermore, the insertion of cloned DNA into the cloning site of M13mp2 leads to a loss of the α-complementation and therefore the plaques produced are white (Sanger et al., 1980).

Restriction Endonuclease Digestion of DNA

Ten times concentration stock solutions (sterile) of restriction endonuclease buffers were prepared. The compositions of the buffers was as outlined for the assay conditions in the New England, Biolabs, Inc. chemical catalogue. One tenth volume of stock solution was added to the DNA solution and restriction endonuclease was added to give a ratio of ~0.5 units enzyme per 1 µg DNA (one unit is defined as the amount of enzyme required to produce a complete digest of 1.0 µg of λ DNA in 60 minutes in a volume of 0.05 mL). The restriction

endonuclease reaction mix was routinely incubated at 37°C (or 67°C in the case of TaqI) for 1 hour.

Preparation of Single Stranded M13mp2 Template

M13mp2 plaques were picked from an agar-overlay plate and cells were grown to stationary phase at 37°C in 1.5 mL of twice strength YT medium. The cells were placed into 1.5 mL capped Eppendorf tubes and centrifuged for 5 minutes. 0.8 mL of the supernatants was removed and placed into a tube containing 200 µL of buffer (2.5 M NaCl, 20% (w/v) polyethylene glycol 6000). This solution was thoroughly mixed and left at room temperature for 30 minutes. The solution was then centrifuged for 10 minutes in an Eppendorf centrifuge. The supernatant was poured out and any remaining supernatant was carefully removed using a drawn out capillary. A small white pellet was visible. The pellet was dissolved in 100 µL $T_{10}E_{0.1}$, pH 7.5. Neutralized phenol (50 µL) was added and the solution was vortexed for 10 seconds, left for 10 minutes, vortexed again and then centrifuged for 1 minute. The aqueous layer was removed and transferred to another Eppendorf tube. The phenol was removed by three extractions with two volumes of diethyl ether. The DNA in the aqueous layer was ethanol precipitated by the sequential addition of 0.1 volume of 5 M NaCl and two volumes of ethanol. The solution was mixed, placed in a bath of cardice-ethanol for one hour and then centrifuged for 10 minutes. The supernatant was removed and the tube dried in vacuo for 5 minutes. The dried pellet was dissolved in 50 µL $T_{10}E_{0.1}$, pH 7.5. Purified single stranded phage was analyzed by electrophoresis on a 0.8% (w/v) agarose gel and detected by staining with ethidium bromide as described below.

Analytical Agarose Gel Electrophoresis

Electrophoresis was carried out on 3 mm thick 0.8% agarose gels using a horizontal slab gel apparatus. Gels were prepared in TEA buffer (40 mM Tris HCl, 1 mM EDTA, 16 mM sodium acetate; pH adjusted to 7.8 with glacial acetic acid). DNA samples (5 µL) were mixed with 15 µL of sample buffer (14.5% sucrose, 0.045% bromophenol blue, 50 mM EDTA) and then loaded into each well by layering under electrophoresis buffer. The nucleic acid was subjected to electrophoresis at room temperature until the bromophenol blue had migrated to the end of the gel. The DNA bands were stained for 15 minutes in 1 µg mL^{-1} ethidium bromide and visualized with ultraviolet light using a transilluminator (Ultra-violet Products, San Gabriel, CA, USA). The gels were photographed using a Polaroid (Model 545) camera and film (high speed type 57). Standard size markers of λ cut with HindIII were included where required.

Preparative Agarose Gel Electrophoresis: Preparation of Purified DNA Fragments Using Hydroxylapatite

Purified pIY1 (Young et al., 1978) with a concentration of ~500 µg mL^{-1} was digested with EcoRI restriction endonuclease. The digested DNA was electrophoresed and the gel stained with ethidium bromide. The plasmid vector (pSF2124) band and the insert band (~1.6 Mdal) were visualized using a hand held shortwave ultraviolet lamp. The band containing the cloned fragment was cut out of the gel with a razor blade and the DNA recovered by electroelution onto hydroxylapatite (Bernardi, 1971). The hydroxylapatite was transferred to a 5 mL sterile plastic syringe. The DNA was eluted from the hydroxylapatite using 0.4 M sodium phosphate, pH 7.0 buffer

containing 1 μg mL^{-1} ethidium bromide. Fractions containing DNA were pooled and the ethidium bromide was removed by 5 extractions with an equal volume of isopropanol and the phosphate buffer removed by dialysis against two changes of STE buffer (2 mM Tris HCl, 2 mM EDTA, 1 mM NaCl, pH 7.5). The DNA concentration was estimated by measurement of ultraviolet absorbance at 260 and 280 nm.

Internal primers used for sequencing the ndh gene were generated by restriction endonuclease digestion and purified using the same method as described above.

Protocol for DNA Sequencing

The primers used were the 26 bp EcoRI/BamHI fragment prepared from a double digest of pSP14 according to the procedure outlined in Anderson et al. (1980) and the internal primers described in the text. The single stranded templates were prepared from M13 clones as described in the previous sections. The sequencing procedure used is outlined below:

(a) A primer/template mix was prepared for hybridization and was composed of:

5 μL M13 template (~0.4 μg DNA)

2 μL primer

1 μL Hin (10x) buffer (0.1 M Tris HCl, 0.1 M $MgCl_2$, 0.5 M NaCl, 10 mM dithiothreitol, pH 7.5)

2 μL sterile distilled water.

Each component of the primer/template mix was added to a capillary, mixed and the capillary sealed. The capillary was then added to a bath of boiling water for three minutes and slowly cooled to 30°C.

(b) The capillary was opened and 2 µL aliquots of the primer/template were each added to four siliconized glass tubes. To each of the four tubes 1 µL of the following solutions was added:

A tube; (5 µL of 0.7 mM ddATP, 1 µL Hin (10x), 1 µL of 0.02 mM dATP and 1 µL [α-^{32}P]dATP).

The other tubes had the same additions except the ddNTPS added were of the following concentrations: 1.5 mM ddGTP, 1 mM ddCTP and 2 mM ddTTP. The solution was mixed by repeatedly taking up the solution and expelling it from the capillary into a siliconized tube.

(c) dNTP mixes were prepared from stocks of 20 mM dNTP (in $T_{10}E_{0.1}$, pH 7.5). The 0.5 mM working stocks were made fresh. The composition of the dNTP mixes were as follows:

	dTTP	dCTP	dGTP	dATP
0.5 mM dTTP	1 µL	20 µL	20 µL	20 µL
0.5 mM dCTP	20 µL	1 µL	20 µL	20 µL
0.5 mM dGTP	20 µL	20 µL	1 µL	20 µL
50 mM Tris HCl, 1 mM EDTA, pH 7.5	5 µL	5 µL	5 µL	5 µL

To 4 µL of each dNTP^{o} mix was added 0.8 µL of Klenow (DNA-polymerase, enzyme according to Klenow, Boehringer-Mannheim, 125 units/50 µL). This solution was mixed and 1.2 µL was immediately added to each reaction capillary and incubated for 15 minutes at room temperature.

(d) To each reaction capillary was added 1 µL dATP (0.5 mM) solution which was a 'chase'. The solutions were mixed and then the capillaries incubated for 15 minutes at room temperature.

(e) 5 µL of formamide dye mix (100 mL deionized formamide, 0.3 g xylene cyanol FF, 0.3 g bromophenol blue and 20 mM EDTA) was added to each reaction tube. The dye was mixed with the contents of the capillary and expelled into the siliconized tube. The tubes were then placed into a boiling water bath for 5 minutes. In the case of experiments in which the primer was cleaved, endonuclease was added and the mixture was incubated for 30 minutes at 37°C prior to the addition of dye mix. Approximately 2 µL was added to each track of a denaturing polyacrylamide gel.

Polyacrylamide Gel Electrophoresis

Thin acrylamide gels used for DNA sequencing were prepared according to the methods outlined in Sanger and Coulson (1978). The urea/acrylamide stock solution used for the gels was prepared as follows: 288 g of urea, 1.8 g bisacrylamide and 34.2 g acrylamide were dissolved at 45°C in distilled water to give a volume of 500 mL. 20 g of amberlite MB-1 resin was added and gently stirred for 30 minutes. The solution was then filtered twice through Whatman No 1 filter papers, 60 mL of ten times strength TBE buffer (1.12 M Tris HCl, 0.89 M boric acid, 0.025 M EDTA, pH 8.3) was added and the volume made up to 600 mL with distilled water. The final composition of the stock solution was (8 M urea, 6% (w/v) acrylamide, 0.11 M Tris HCl, 0.09 M boric acid, 2.5 mM EDTA, pH 8.3).

The gels were formed between two 20 x 38 x 0.4 cm glass plates (0.4 mm spacers between them), one with a notch 16.5 x 2 cm cut out from the top to allow contact between the gel and the upper compartment of the apparatus (Sanger and Coulson, 1978).

Usually 2 µL of the sequencing reaction mixture in dye mix was loaded onto each of two polyacrylamide gels which were

electrophoresed at 1500 volts for 2.2 and 4.5 hours respectively, and the glass plates separated. The gel was covered with Saran wrap and autoradiographed using Kodak X-omat H film. In the early experiments autoradiography was performed at -60°C but in later work the gels were fixed in 10% (v/v) acetic acid in water prior to autoradiography at room temperature.

RESULTS AND DISCUSSION

In early 1979 the project for the sequencing of the cloned *ndh* gene (Young *et al.*, 1978) was initiated. At that time one of the strategies used for sequencing was to use double stranded restriction fragments as primers and a single stranded template. This strategy had been successfully employed for the sequencing of bacteriophage ØX174 where single stranded template is readily available (Sanger, Air, Barrell, Brown, Coulson, Fiddes, Hutchison, Slocombe and Smith, 1977). With the development of the M13 cloning vector M13mp2 this approach could be extended to other DNA's and it was utilized in the present work. It soon became clear, however, that high quality sequence data could be obtained more easily by randomly cloning restriction fragments into M13 vectors and using a 'universal' flanking primer to initiate chain extension across the cloned fragment. This approach because of its random nature requires adequate computer programs to search for overlaps and to enable a complete sequence to be assembled. The lack of suitable computer facilities precluded the use of this approach at that time in this laboratory. Sequencing experiments of this type were carried out in

Cambridge U.K. in early 1980 by Dr I.G. Young whilst on study leave in Dr F. Sanger's laboratory.

The sequencing strategy for the ndh gene using internal primers is outlined below. Plasmid pIY1 (Young et al., 1978), which carries the cloned ndh fragment (designated as fragment 1-1), and the double-stranded RF of M13mp2 were digested separately with EcoRI endonuclease, ligated together, and transfected into strain 71-18 (Messing et al., 1977; Sanger et al., 1980). Plaques which were white on indicator plates containing X-gal were propagated, and the replicative forms of the hybrid phages isolated. Two hybrid phages carrying the ndh fragment cloned in opposite orientations (designated A and B) were obtained. Since mature M13 virus carries single stranded DNA, the two different hybrid phages provided a source of both complementary strands of the ndh gene.

During subsequent cultivation, the size of the DNA of the progeny phages was monitored by agarose gel electrophoresis to detect the presence of deleted forms. It was found that the two different hybrid phages differed in stability. Only one orientation of the cloned fragment resulted in instability of the hybrid phage (orientation B), suggesting that the sequence of the insert may be important in determining the stability of hybrid phages. Both phages could be grown without significant deletion if plaque-purified before use, and if grown on a small scale.

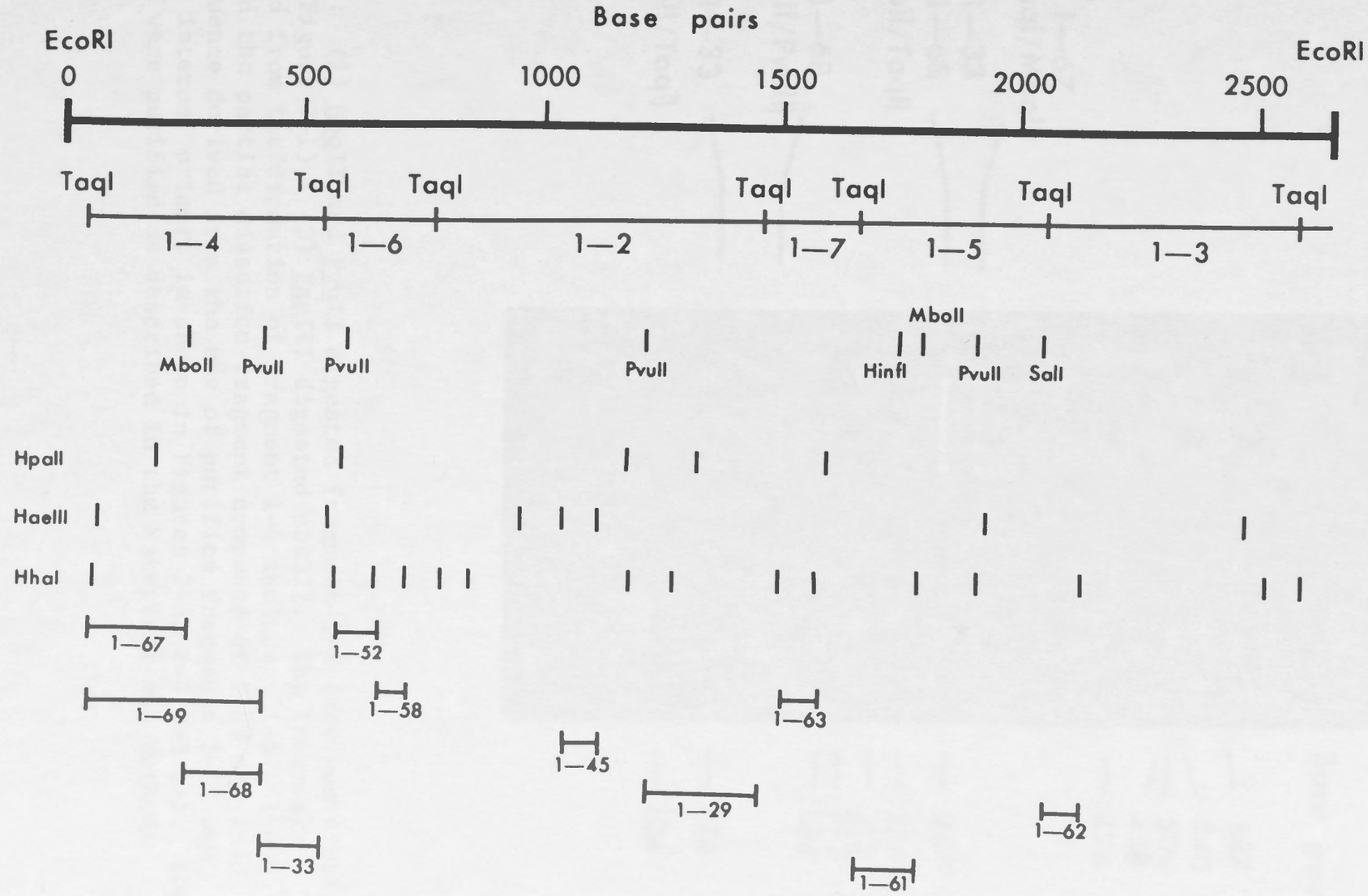
The cloned fragment containing the ndh gene (fragment 1-1) was purified by hydroxylapatite chromatography as described in the Materials and Methods section. Using different restriction endonucleases a preliminary restriction map was constructed (Figure 2-1). Purified fragment 1-1 was digested with a series of

restriction endonucleases with 5 or 6 base recognition sites (eg TaqI PvuII, MboII, SalI, PvuI and HinfI). The order of the fragments was established by double digestions with these enzymes. TaqI digestion of purified fragment 1-1 gave 6 major fragments which were purified by agarose gel electrophoresis and hydroxylapatite chromatography. The order of the TaqI digestion fragments (numbered 1-2 to 1-7) was determined by using double digests with the restriction endonucleases listed above. To produce small restriction fragments for use as primers, restriction endonucleases with 4 base recognition sites were used to cleave each of the purified TaqI fragments. The restriction fragments produced by these digestions were each given a numerical designation (1-n) and their respective nucleotide chain lengths were calculated by polyacrylamide gel electrophoresis (Maniatis, Jeffrey and deSande, 1975). ØX174 and pBR322 DNA which had been digested with HaeIII endonuclease were used as size standards. Primers 1-67, 1-68 and 1-33 are shown as an example in Figure 2-2.

A summary of the sequence data obtained using internal primers is illustrated in Figure 2-3. All the primers were cleaved with restriction endonuclease prior to polyacrylamide electrophoresis, as shown in the restriction map (Figure 2-1), except the very short primers (1-52, 1-58, 1-45, 1-61 and 1-62).

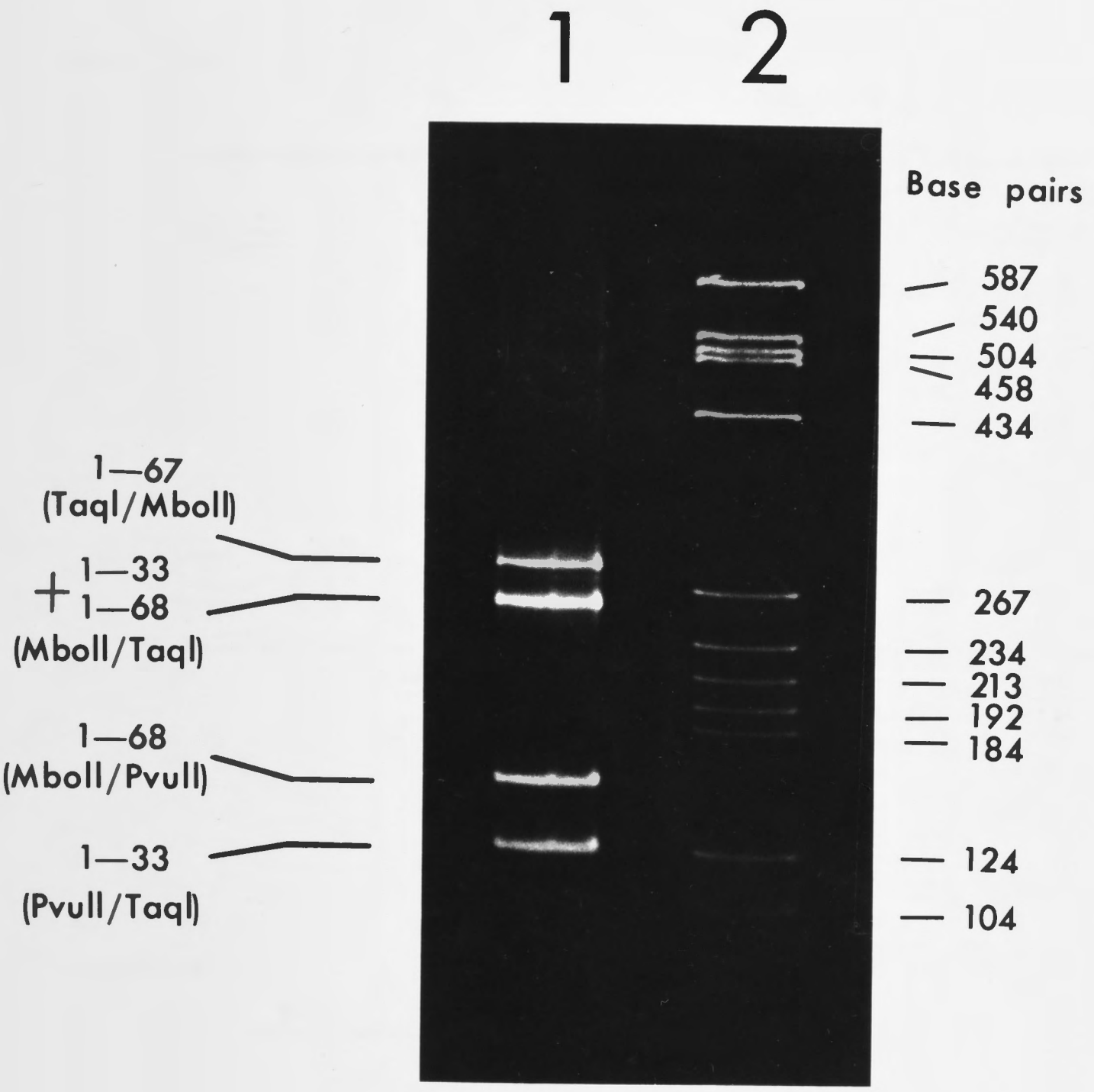
The 'internal' primer sequencing method using primers described above proved difficult to apply in practice. The sequence data obtained generally showed a higher level of ambiguity than expected, and long sequence readings were not obtained. Later developments indicated that the problems were probably due to impure primers leading to inhibition of the copying of the template by the DNA polymerase I.

Figure 2-1. Restriction Endonuclease Map of the Cloned Fragment 1-1 Containing the Ndh Structural Gene



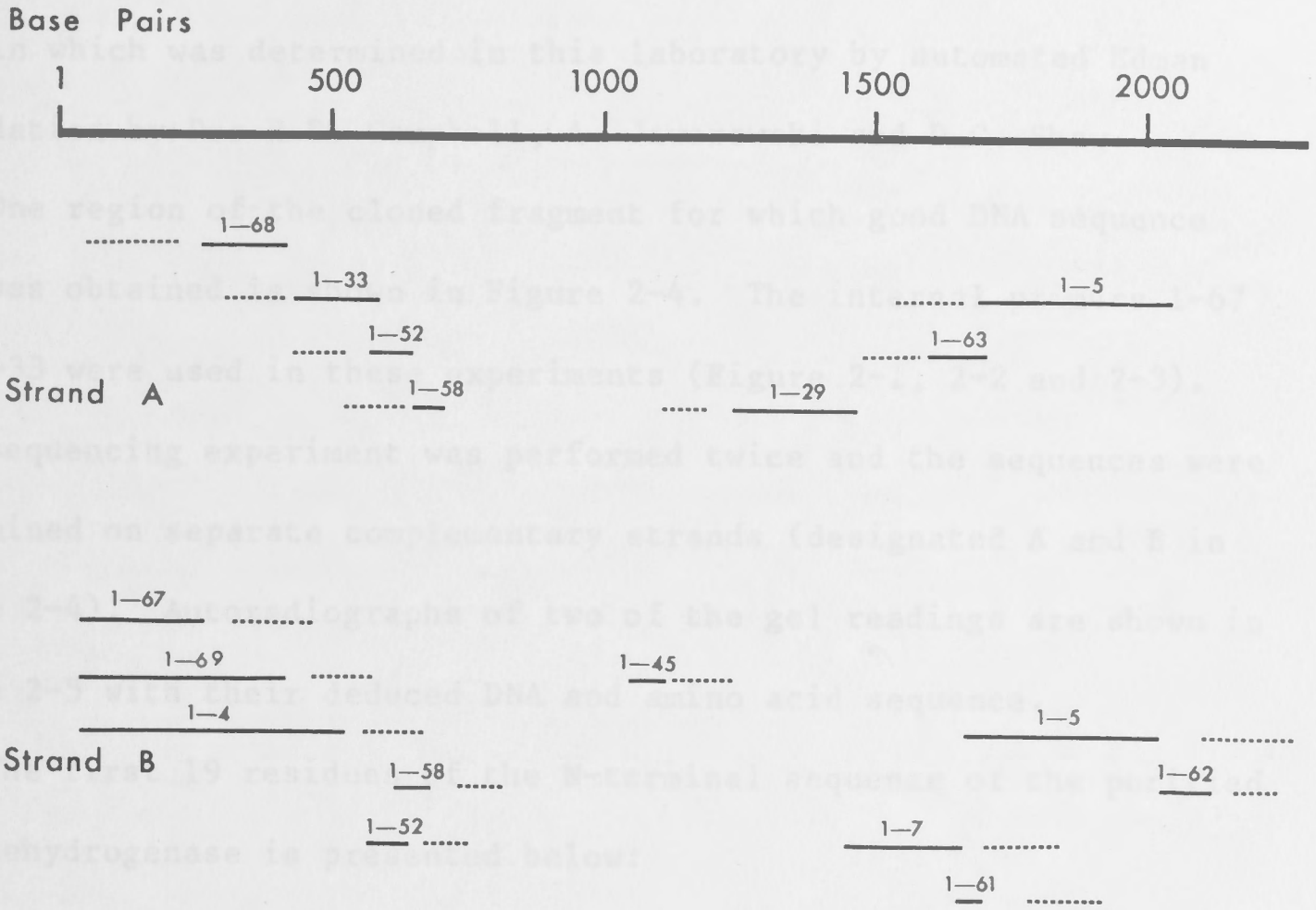
The restriction map was determined as described in the text. Endonuclease recognition sites are indicated by vertical bars. The TaqI fragments, numbered 1-2 to 1-7, were purified and further cleaved to produce the fragments listed at the bottom of the figure. These fragments were purified as described in the Materials and Methods section and used as internal primers in sequencing experiments.

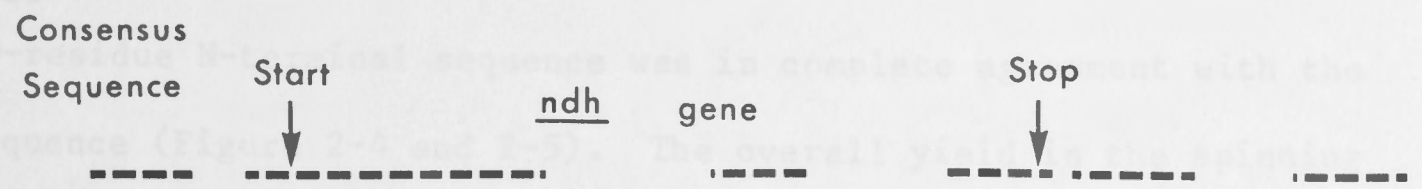
Figure 2-2. Analytical Polyacrylamide Gel Electrophoresis of Endonuclease Digested Fragment 1-4: Production of Internal Primers



Samples: (1) MboII and PvuII digested fragment 1-4 (see restriction map in Figure 2-1), (2) HaeIII digested pBR322. The fragments produced from the digestion of fragment 1-4 include: 1-67, 1-68, 1-33 and the partial digestion fragment composed of 1-68 and 1-33. DNA sequence derived from the use of purified fragments 1-33 and 1-67 as internal primers is shown in Figures 2-3, 2-4 and 2-5. The primers were purified as described in the Materials and Methods section.

Figure 2-3. Summary of Sequence Data Obtained Using Internal Primers and M13 Clones Carrying the Whole Ndh Fragment as Template





The regions where sequence data was obtained are represented by dotted lines. Some primers were cleaved with endonucleases prior to electrophoresis. The endonucleases used are shown in the restriction map in Figure 2-1. See text and Materials and Methods section for more details.

The sequence data obtained using internal primers was examined for sequences corresponding to the N-terminal coding sequence of the protein which was determined in this laboratory by automated Edman degradation by Drs H.D. Campbell, A. Jaworowski and D.C. Shaw.

One region of the cloned fragment for which good DNA sequence data was obtained is shown in Figure 2-4. The internal primers 1-67 and 1-33 were used in these experiments (Figure 2-1, 2-2 and 2-3). Each sequencing experiment was performed twice and the sequences were determined on separate complementary strands (designated A and B in Figure 2-4). Autoradiographs of two of the gel readings are shown in Figure 2-5 with their deduced DNA and amino acid sequence.

The first 19 residues of the N-terminal sequence of the purified NADH dehydrogenase is presented below:

Thr-Thr-Pro-Leu-Lys-Lys-Ile-Val-Ile-Val-Gly-Gly-Gly-
$\begin{matrix}\text{Ala}\\\text{Ser}\end{matrix}$-Gly-Gly-Leu-Glx-Met

The 19-residue N-terminal sequence was in complete agreement with the DNA sequence (Figure 2-4 and 2-5). The overall yield in the spinning cup for the first residue (threonine) was approximately 70%, which is close to the expected maximum yield of ~80%. This indicates that the initiating formylmethionine has been largely or completely removed by post-translational processing.

One puzzling feature of the DNA sequence at this time was the absence of a suitable initiation codon. There were no suitable AUG or GUG codons upstream from the N-terminal sequence. This ruled out the possibility that the dehydrogenase used a signal peptide for membrane assembly and suggested that post-translational processing probably involved the removal of formylmethionine. This indicated that the initiation codon would be UUG which had not previously been

Figure 2-4. The DNA Sequence of the Region Encoding the N-terminus of the Ndh Protein

The direction of the gel readings are indicated by arrows. The consensus sequence is in large type. The initiation codon UUG is underlined as is the ribosome binding site. The letters A and B indicate the complementary strands of the cloned ndh fragment. Examples of some gel reading autoradiographs covering this region are shown in Figure 2-5.

1—67 B

→ A T G T T A A T A A C C A T T A A T T A A C A A T T G G T T

A N G N T A A T A A C C A T T A A T T A A C A A T T G G T T ←
C C

T G T T A A T A A C C A T T A A T T A A C A A T T G G T T ←

ATGTTAATAACCATTAATTAACAATTGGTT

1—67 B

→ G N C A C G T T G A C T A C G

A A T A A A T T T A A G G G G G T C A C G T T G A C T A C G

A A T A A A T T T A A G G G G G T C A C G T T G A C T A C G ←

A A T A A A T N T A A G G G G G T C A C G T T G A C T A C G ←

Met Thr Thr

AATAAATTTAAGGGGGTCACGTTGACTACG

→ C C A T T G A A A A A G A T T G T G A T T G T C G G C G G C

→ C C A T T G A A A A A G A T T G T G A T T G T C G G C G G C

C C A T T G A A A A A G A T T G A T T G A T T G C C G G A G G C ←
G T T C A

C C A T T G A A A A A G A T T G T G A T T G T C G ← 1—33 A

Pro Leu Lys Lys Ile Val Ile Val Gly Gly

CCATTGAAAAAGATTGTGATTGTCGGCGGC

→ G G T G N T G G T G G G C T G G A A

→ G G T G C T G G T G G G C T

G G
A G T G C T G G T G T T C T ← 1—33 A

Gly Ala Gly Gly Leu Glu

GGTGCTGGTGGGCTGGAA

Figure 2-5. Autoradiographs of Acrylamide Gel Electrophoresis from Experiments which Identified the N-terminal Coding Region

Gel A

Gel B

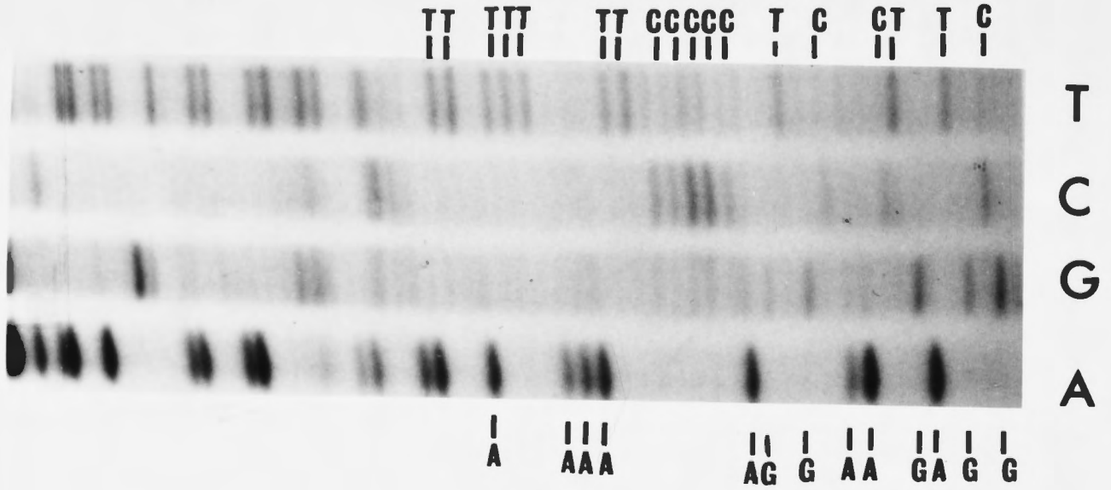
A A T A A A T T T A A G G G G G T C A C G T T G A C T A C G C

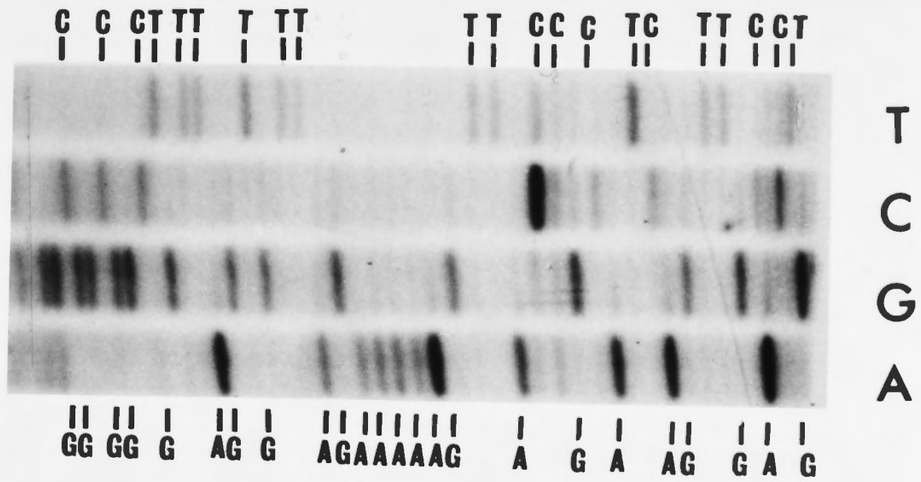
Met Thr Thr Pro Leu Lys Lys Ile Val Ile Val Gly Gly

G T C A C G T T G A C T A C G C C A T T G A A A A A G A T T G T G A T T G T C G G C G G C

primer 1—33 **PvuII** **A**

primer 1—67 **MboII** **B**





The initiation codon and the ribosome binding site are underlined. The letters A and B indicate the complementary strands of the cloned ndh fragment. The primers used, 1-33 and 1-67, are described in the text and in Figures 2-1, 2-2 and 2-3. The endonucleases PvuII and MboII were used to cleave the primers.

shown to be a normal initiation codon. The presence of a ribosome binding site (Shine and Dalgarno, 1974;1975) four bases upstream from the UUG gave additional support to this hypothesis.

Since at the time the use of UUG as an initiation codon was very novel, additional experiments involving *in vitro* transcription/translation were carried out in this laboratory which verified that UUG does code for N-formylmethionine in this instance (Poulis, Shaw, Campbell and Young, 1981).

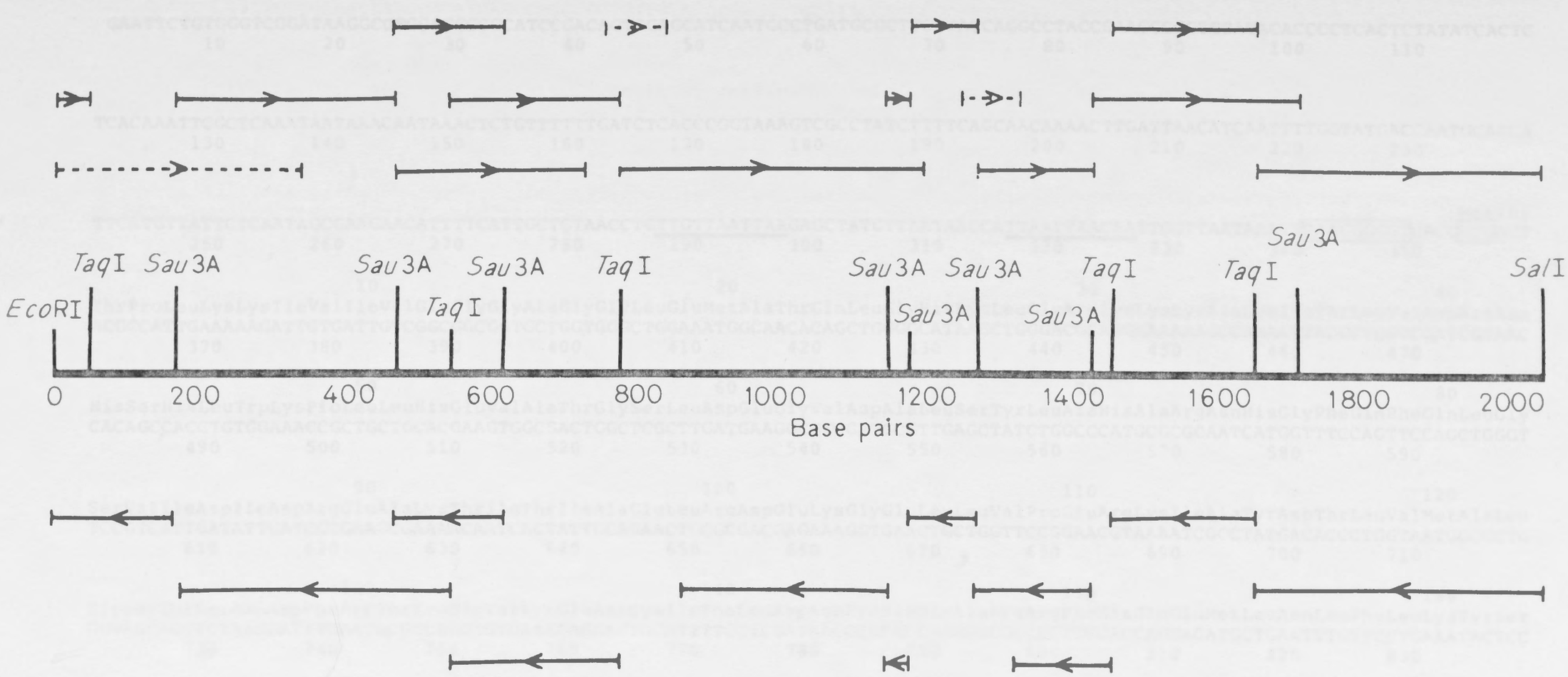
Overall, the problem of the high level of ambiguity in the DNA sequence data suggested that an alternative sequencing strategy should be attempted. The method chosen was to randomly clone fragments into M13mp2 and to use a universal primer for sequencing experiments. These sequencing experiments were carried out by Dr I.G. Young whilst on study leave in Cambridge, U.K., where suitable computer facilities were available.

Plasmid pIY1 (Young *et al.*, 1978) was digested with *Eco*RI endonuclease and the cloned *ndh* fragment was purified by gel electrophoresis using low-melting agarose (Sanger *et al.*, 1980). The restriction endonucleases *Sau*3A and *Taq*I were used to generate the small fragments for subcloning into M13. Fragments derived by digestion with *Sau*3A were cloned directly into the *Bam*HI site of the vector M13mWJ22 (Rothstein *et al.*, 1980). *Bam*HI linkers were used to clone *Taq*I fragments into the same vector (ibid.). The chain-termination method of Sanger *et al.* (1977) was used for sequencing the M13 clones. The 26 bp primer used (Anderson *et al.*, 1980) anneals adjacent to the *Eco*RI site of M13mp2 and related vectors, and directs the primed synthesis into the cloned fragments. Individual M13 clones were propagated on a 1 mL scale and the template DNA was

prepared after precipitation of the phage using poly(ethyleneglycol) (Sanger et al., 1980). The M13 clones carrying random short fragments were screened initially by carrying out the normal sequencing reaction with only one dideoxynucleotide (usually ddT). In this case the positions of thymines in the DNA sequences were determined and used as a basis for comparison of the various clones. By recognizing the sequence of the vector this technique enabled the size of the inserts to be determined as well as their relationship with each other and with other sequence data. The random sequence data determined on both complementary strands were assembled into a complete sequence using the computer programs described by Staden (1977; 1980). A summary of the gel readings used to assemble the sequence is given in Figure 2-6 (taken from the reference; Young, Rogers, Campbell, Jaworowski and Shaw, 1981). The sequence itself is shown in Figure 2-7 (taken from Young et al., 1981). Examination of both strands of the DNA sequence showed the presence of only one reading frame compatible with the subunit M_r of 47000 determined for the purified enzyme (Jaworowski et al., 1981b). This reading frame is shown in Figure 2-7 and predicts a protein sequence of 434 residues with an M_r of 47200. The amino acid analysis (performed by Drs H.D. Campbell, A. Jaworowski and D.C. Shaw) of the purified enzyme gave good overall agreement with the predicted amino acid composition (Young et al., 1981). In other studies (Jaworowski et al., 1981b) the 16-residue C-terminal cyanogen bromide peptide predicted from the nucleotide sequence has been isolated which further confirmed the reading frame.

The nucleotide sequence presented in Table 2-7 is discussed in the next section of this Chapter. Since some time has elapsed from

Figure 2-6. Summary of DNA sequence Data Obtained from the M13 Clones



The restriction sites for TaqI and Sau3A in the ndh gene are shown. The lines above and below the restriction map indicate the various gel readings obtained for the two DNA strands. The solid lines are used for data that was obtained from M13 carrying subfragments which were sequenced using an external primer. The dotted lines indicate that the data was obtained with M13 clones carrying the whole ndh fragment as template. In this case, for sequences within the gene, internal primers were used. The minimum overlap in the gel readings used to construct the sequence was 17 nucleotides.

Figure 2-7. The Nucleotide Sequence of the Ndh Gene

```
GAATTCTGTGGGTCGGATAAGGCGTCCACGCCGCATCCGACAGTCGAGCATCAATGCCTGATGCGCTTCTTATCAGGCCTACCGAACGCCCTGCATACACCCCTCACTCTATATCACTC
        10        20        30        40        50        60        70        80        90       100       110

TCACAAATTCGCTCAAATAATAAACAATAAACTCTGTTTTTTGATCTCACCCGGTAAAGTCGCCTATCTTTTCAGCAACAAAACTTGATTAACATCAATTTTGGTATGACCAATGCACCA
      130       140       150       160       170       180       190       200       210       220       230

                                                                                                          Met Thr
TTCATGTTATTCTCAATAGCGAAGAACATTTTCATTGCTGTAACCTGTTGTTAATTAAGAGCTATGTTAATAACCATTAATTAACAATTGGTTAATAAATTTAAGGGGGTCACGTTGACT
      250       260       270       280       290       300       310       320       330       340       350

                    10                  20                  30                  40
ThrProLeuLysLysIleValIleValGlyGlyGlyAlaGlyGlyLeuGluMetAlaThrGlnLeuGlyHisLysLeuGlyArgLysLysLysAlaLysIleThrLeuValAspArgAsn
ACGCCATTGAAAAAGATTGTGATTGTCGGCGGCGGTGCTGGTGGGCTGGAAATGGCAACACAGCTGGGGCATAAGCTGGGACGCAAGAAAAAAGCCAAAATTACGCTGGTCGATCGTAAC
      370       380       390       400       410       420       430       440       450       460       470

                    50                  60                  70                  80
HisSerHisLeuTrpLysProLeuLeuHisGluValAlaThrGlySerLeuAspGluGlyValAspAlaLeuSerTyrLeuAlaHisAlaArgAsnHisGlyPheGlnPheGlnLeuGly
CACAGCCACCTGTGGAAACCGCTGCTGCACGAAGTGGCGACTGGCTCGCTTGATGAAGGCGTCGATGCGTTGAGCTATCTGGCCCATGCGCGCAATCATGGTTTCCAGTTCCAGCTGGGT
      490       500       510       520       530       540       550       560       570       580       590

                    90                  100                 110                 120
SerValIleAspIleAspArgGluAlaLysThrIleThrIleAlaGluLeuArgAspGluLysGlyGluLeuLeuValProGluArgLysIleAlaTyrAspThrLeuValMetAlaLeu
TCCGTCATTGATATTGATCGTGAAGCGAAAACAATCACTATTGCAGAACTGCGCGACGAGAAAGGTGAACTGCTGGTTCCGGAACGTAAAATCGCCTATGACACCCTGGTAATGGCGCTG
      610       620       630       640       650       660       670       680       690       700       710

                    130                 140                 150                 160
GlySerThrSerAsnAspPheAsnThrProGlyValLysGluAsnCysIlePheLeuAspAsnProHisGlnAlaArgArgPheHisGlnGluMetLeuAsnLeuPheLeuLysTyrSer
GGTAGCACCTCTAACGATTTCAATACGCCAGGTGTCAAAGAGAACTGCATTTTCCTCGATAACCCGCACCAGGCGCGTCGCTTCCACCAGGAGATGCTGAATTTGTTCCTGAAATACTCC
      730       740       750       760       770       780       790       800       810       820       830

                    170                 180                 190                 200
AlaAsnLeuGlyAlaAsnGlyLysValAsnIleAlaIleValGlyGlyGlyAlaThrGlyValGluLeuSerAlaGluLeuHisAsnAlaValLysGlnLeuHisSerTyrGlyTyrLys
GCCAACCTGGGCGCGAATGGCAAAGTGAACATTGCGATTGTCGGCGGCGGCGCGACGGGTGTAGAACTCTCCGCTGAATTGCACAACGCGGTCAAGCAACTGCACAGCTACGGTTACAAA
      850       860       870       880       890       900       910       920       930       940       950

                    210                 220                 230                 240
GlyLeuThrAsnGluAlaLeuAsnValThrLeuValGluAlaGlyGluArgIleLeuProAlaLeuProProArgIleSerAlaAlaAlaHisAsnGluLeuThrLysLeuGlyValArg
GGCCTGACCAACGAAGCCCTGAACGTAACGCTGGTAGAAGCGGGAGAACGTATTTTGCCTGCGTTACCGCCACGTATCTCTGCTGCGGCCCACAACGAGCTAACGAAACTTGGCGTTCGC
      970       980       990      1000      1010      1020      1030      1040      1050      1060      1070
```

```
                    250                 260                 270                 280
ValLeuThrGlnThrMetValThrSerAlaAspGluGlyGlyLeuHisThrLysAspGlyGluTyrIleGluAlaAspLeuMetValTrpAlaAlaGlyIleLysAlaProAspPheLeu
GTGCTGACGCAAACCATGGTCACCAGTGCTGATGAAGGCGGCCTGCACACTAAAGATGGCGAATATATTGAGGCTGATCTGATGGTATGGGCAGCCGGGATCAAAGCGCCAGACTTCCTG
      1090      1100      1110      1120      1130      1140      1150      1160      1170      1180      1190

                    290                 300                 310                 320
LysAspIleGlyGlyLeuGluThrAsnArgIleAsnGlnLeuValValGluProThrLeuGlnThrThrArgAspProAspIleTyrAlaIleGlyAspCysAlaSerCysProArgPro
AAAGATATCGGTGGTCTTGAAACTAACCGTATCAACCAGCTGGTGGTGGAACCGACGCTGCAAACCACCCGCGATCCAGACATTTACGCTATTGGCGACTGCGCGTCATGCCCGCGTCCG
      1210      1220      1230      1240      1250      1260      1270      1280      1290      1300      1310

                    330                 340                 350                 360
GluGlyGlyPheValProProArgAlaGlnAlaAlaHisGlnMetAlaThrCysAlaMetAsnAsnIleLeuAlaGlnMetAsnGlyLysProLeuLysAsnTyrGlnTyrLysAspHis
GAAGGGGGCTTTGTTCCGCCGCGTGCTCAGGCTGCACACCAGATGGCGACTTGCGCAATGAACAACATTCTGGCGCAGATGAACGGTAAGCCGCTGAAAAATTATCAGTATAAAGATCAT
      1330      1340      1350      1360      1370      1380      1390      1400      1410      1420      1430

                    370                 380                 390                 400
GlySerLeuValSerLeuSerAsnPheSerThrValGlySerLeuMetGlyAsnLeuThrArgGlySerMetMetIleGluGlyArgIleAlaArgPheValTyrIleSerLeuTyrArg
GGTTCGCTGGTATCGCTGTCGAACTTCTCCACCGTCGGTAGCCTGATGGGTAACCTGACGCGCGGCTCAATGATGATTGAAGGACGAATTGCGCGCTTTGTATATATCTCGCTATACCGA
      1450      1460      1470      1480      1490      1500      1510      1520      1530      1540      1550

                    410                 420                 430
MetHisGlnIleAlaLeuHisGlyTyrPheLysThrGlyLeuMetMetLeuValGlySerIleAsnArgValIleArgProArgLeuLysLeuHis
ATGCATCAGATTGCGCTGCATGGTTACTTTAAAACCGGATTAATGATGCTGGTGGGGAGTATTAACCGCGTTATCCGTCCGCGTTTGAAGTTGCATTAATCGACGTACACTGGCGGATGT
      1570      1580      1590      1600      1610      1620      1630      1640      1650      1660      1670

GGCATAAACGCCTCATCCGCCCTTGAGGAACAGCGCGATCGGCAGCCGCGTTGTATCAGGCATCCTTTCAGACTCCTCCGAATCCTTAAGTATTTCCAGCCATTCCCGCGCTTTTCATCT
      1690      1700      1710      1720      1730      1740      1750      1760      1770      1780      1790

TCTGTCTGATAGCTGCTTTTCTCCTTCGCTTGCATGATTGGCATAACTGCAAAGAAGGAGGTGTTCCCGTGAATAAATCAATGTTGGCGGGTATCGGGATTGGTGTCGCAGCTGCGCTGG
      1810      1820      1830      1840      1850      1860      1870      1880      1890      1900      1910

GCGTAGCGGCAGTGGCCAGTCTGAACGTGTTTGAACGGGGCCCGCAATACGCTCAGGTTGTTTCTGCAACCCCAATCAAGGAAACGGTTAAAACACCGCGTCAGGAGTGTCGCAACGTCA
      1930      1940      1950      1960      1970      1980      1990      2000      2010      2020      2030

CAGTGACCCATCGTCGAC
      2050
```

The region shown covers most of the ndh fragment, starting at the EcoRI site at one end and terminating at a unique SalI within the fragment. Two pairs of inverted repeats present in the nucleotide sequence (287-297, 316-326; 1671-1682, 1689-1700) are underlined. The possible ribosome binding site and UUG initiation codon are indicated by the boxes. The d representing deoxy and the hyphens representing phosphodiester linkages have been omitted. The prediced amino acid sequence is aligned above the nucleotide sequence.

the time of the publication of the sequence (mid-1981), the discussion has integrated subsequent work by others.

FEATURES OF THE NUCLEOTIDE SEQUENCE

Transcription Control Sequences

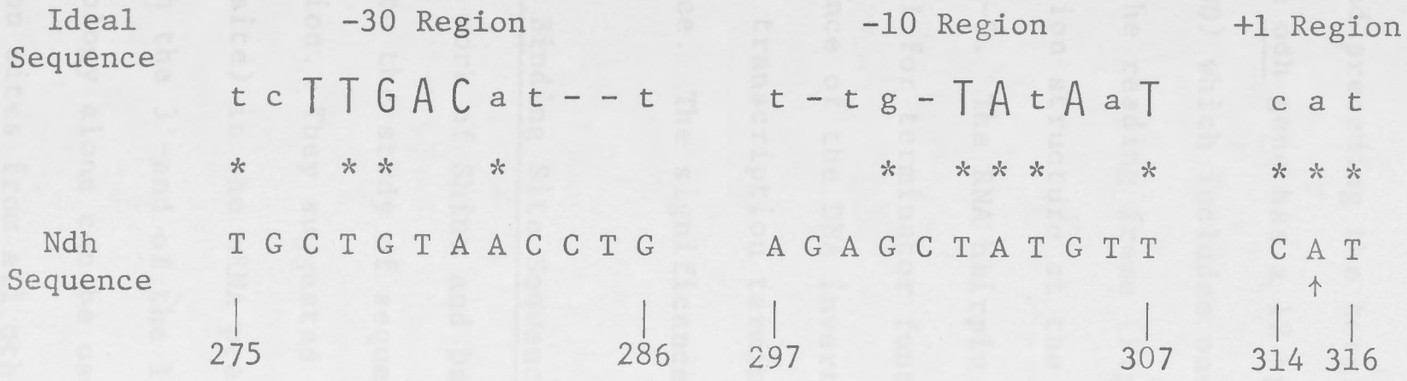
Pribnow (1975) and Schaller, Gray and Herrmann (1975) after comparing a few promoter sequences noted that each contained a seven base pair sequence generally homologous to the sequence TATAATG, which was centred about 10 bp upstream from the mRNA startpoint. Rosenberg and Court (1979) compared the nucleotide sequences of 46 promoter sites recognized by the E. coli RNA polymerase. They concluded that as judged by sequence homology alone, the conserved -10 region clearly extends for some 13 nucleotide pairs and the extent to which particular positions within the region are conserved appears to vary widely.

Sequence similarity has also been noted among promoters in a region about 35 bp preceding the mRNA startpoint (called the -35 or recognition region) (Takanami, Sugimoto, Sugisaki and Okamoto, 1976).

An idealized promoter sequence which is a composite of 112 known promoter sequences (Hawley and McClure, 1983) is presented in Figure 2-8 together with a putative promoter sequence upstream from the initiation codon of the ndh gene.

The gene sequence was also examined for the presence of inverted repeats. An 11-base inverted repeat is present in the promoter region (nucleotides 287-297, 316-326), the significance of which is unclear at present (Figure 2-7).

Figure 2-8. Representation of an Ideal Promoter Sequence: Comparison with a Putative Promoter Sequence of the Ndh Gene



The ideal consensus promoter for E. coli RNA polymerase is derived from a compilation of 112 promoter sequences (Hawley and McClure, 1983). Bases that occur in at least 39% of the promoters are listed. Bases that are >54% conserved are capitalized. An asterisk marks regions of agreement with the ndh sequence. Both of the sequences presented are of the sense strand. The nucleotide corresponding to the likely 5' end of the transcript (+1) is indicated by an arrow.

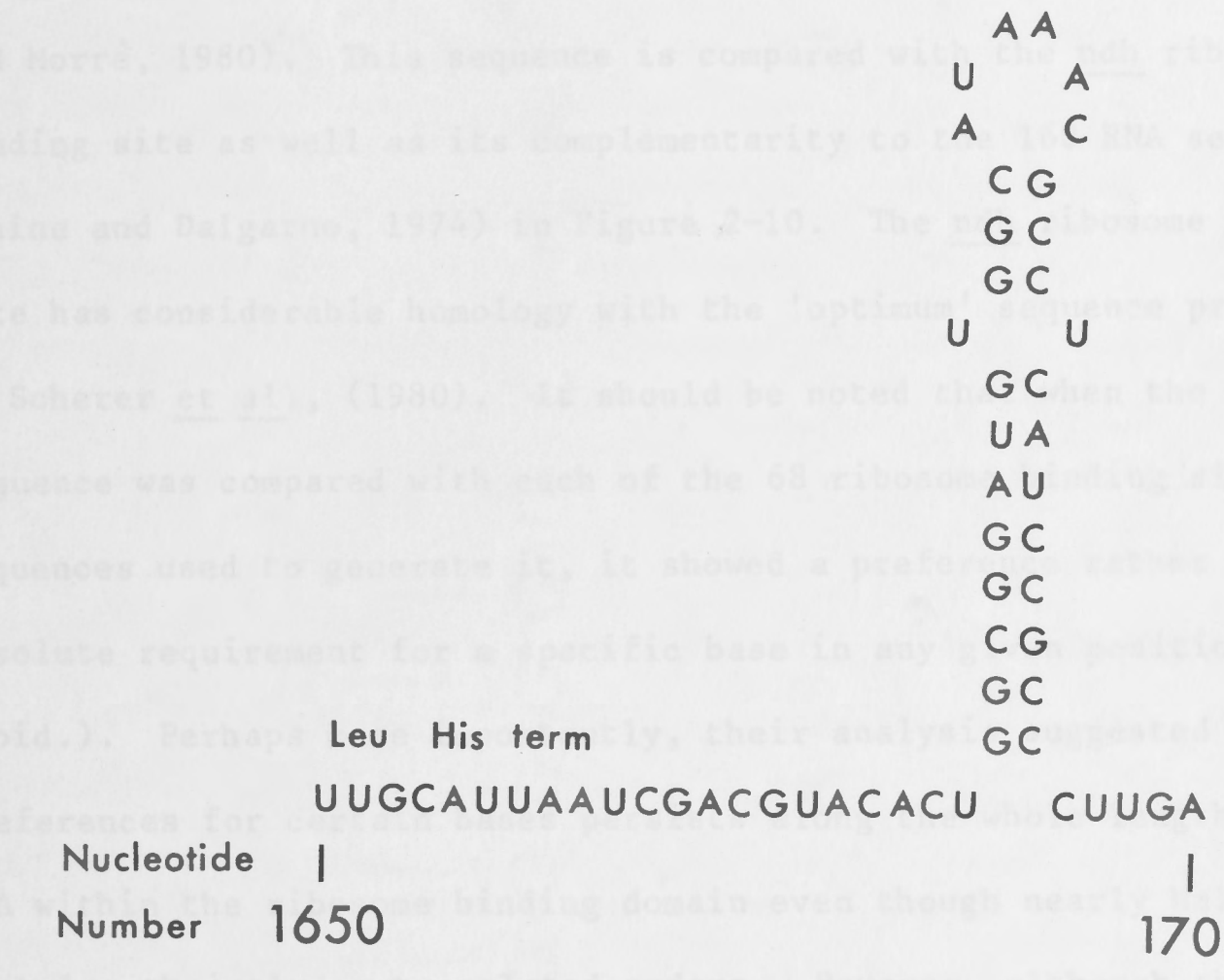
The DNA sequences of a number of sites that encode information for transcription termination have been determined (Biro and Weissman, 1979). Comparison of termination site sequences reveal three common features: (a) a region of hyphenated dyad symmetry (ie an inverted repeat sequence) precedes the termination site, (b) U residues are found at the terminus of the RNA transcript, and (c) G/C rich sequences variable in length (3 to 11 contiguous G/C base pairs) are found preceding the termination site (ibid.).

The ndh gene has a 12 bp inverted repeat (nucleotides 1671-1682, 1689-1700) which includes one mismatch, a short distance after the end of the reading frame (Figure 2-7). A possible RNA transcription termination structure at the 3' end of the ndh gene is illustrated in Figure 2-9. The RNA hairpin (stem and loop) structure that is essential for terminator function is illustrated, and is a direct consequence of the DNA inverted repeat sequence. However, the ndh gene RNA transcription termination structure does not possess a poly-U sequence. The significance of this is unknown.

Ribosome Binding Site Sequence

The work of Shine and Dalgarno (1974;1975) provided a major impetus to the study of sequences involved in the initiation of translation. They suggested that a purine-rich sequence (ribosome binding site) in the mRNA preceding the translation start could base pair with the 3'-end of the 16S RNA of the small ribosomal subunit. This homology alone can be used to distinguish E. coli translational initiation sites from all other sites in tabulated mRNA sequence libraries (Stormo, Schneider, Gold and Ehrenfeucht, 1982a; Stormo, Schneider and Gold, 1982b).

Figure 2-9. A Possible RNA Transcription Termination Structure at the 3' End of the Ndh Gene



The 3' end of the ndh gene has been represented as a sequence of ribonucleotides in a stem and loop secondary structure. The C-terminal histidine is followed by one nonsense codon (term). A G/C-rich sequence forms the stem, however there is not a U-rich region downstream.

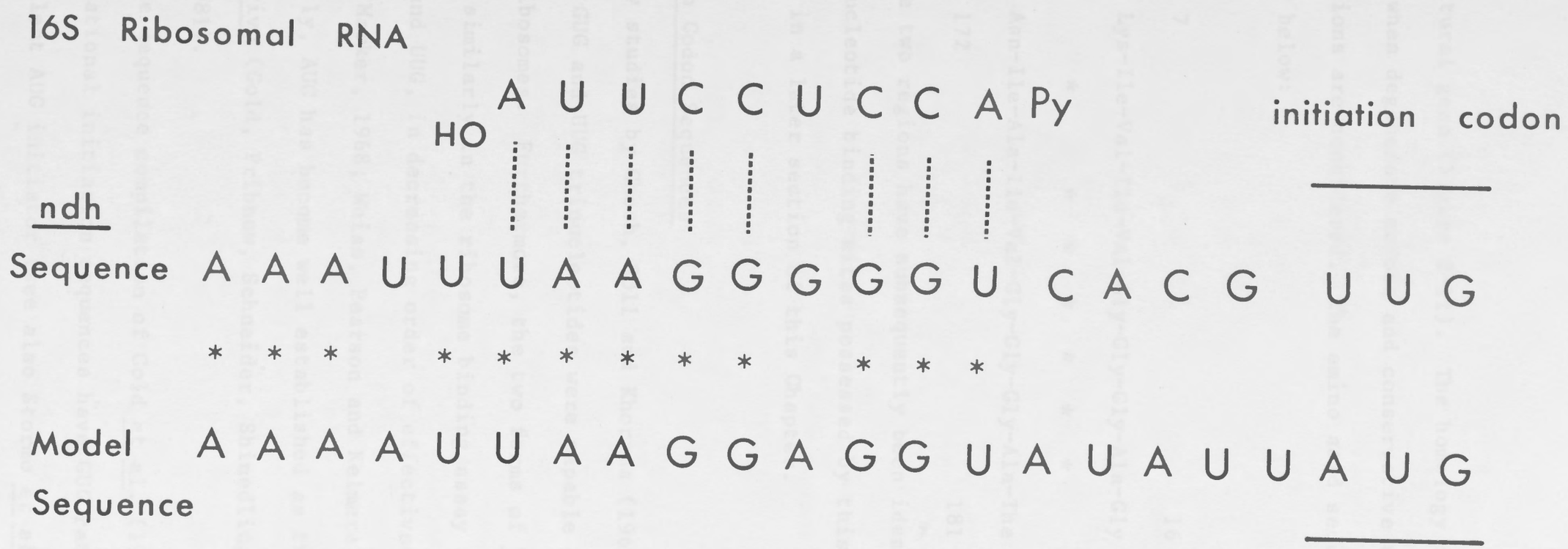
Using an E. coli mRNA sequence database, the sequence of nucleotides that should be present in an mRNA for optimum binding to the E. coli ribosome has been calculated (Scherer, Walkinshaw, Arnott and Morrè, 1980). This sequence is compared with the ndh ribosome binding site as well as its complementarity to the 16S RNA sequence (Shine and Dalgarno, 1974) in Figure 2-10. The ndh ribosome binding site has considerable homology with the 'optimum' sequence proposed by Scherer et al., (1980). It should be noted that when the model sequence was compared with each of the 68 ribosome binding site sequences used to generate it, it showed a preference rather than an absolute requirement for a specific base in any given position (ibid.). Perhaps more importantly, their analysis suggested that preferences for certain bases persists along the whole length of the RNA within the ribosome binding domain even though nearly half of that length includes translated codons. However, although the ndh ribosome binding site matches well with the model mRNA sequence deduced by Scherers et al. (1980) upstream from the initiation codon, less homology is observed in the early translated region (not shown in Figure 2-10). A comparison is listed below:

```
ndh gene      UUGACUACGCCAUUGAAAAAGAU
                ******        ***  **
model         AUGAAAAAAAUUAAAAAACUCAA
sequence          CUC             G
```

Internal Sequence Repeats

One obvious feature of the ndh DNA sequence is an internal sequence repeat of 15 bp at nucleotides 380-394 and 875-889 in the

Figure 2-10. The Ribosome Binding Site of the Ndh Gene



The 3'-terminal sequence of E. coli 16S ribosomal RNA (Shine and Dalgarno, 1974) is shown aligned above a complementary region 5' to the UUG initiation codon of the ndh gene. Complementary base matches are indicated by a dotted line. Aligned below the ndh sequence is a model sequence proposed by Scherer et al. (1980) that represents an mRNA which exhibits optimum binding to the E. coli ribosome. Agreement with the ndh sequence is shown by an asterisk.

ndh structural gene (Figure 2-11). The homology is even more striking when degenerate codons and conservative amino acid substitutions are considered. The amino acid sequence homology is described below:

Residue

Number 7 16

Lys-Ile-Val-Ile-Val-Gly-Gly-Gly-Ala-Gly

* * * * * * *

Asn-Ile-Ala-Ile-Val-Gly-Gly-Gly-Ala-The

172 181

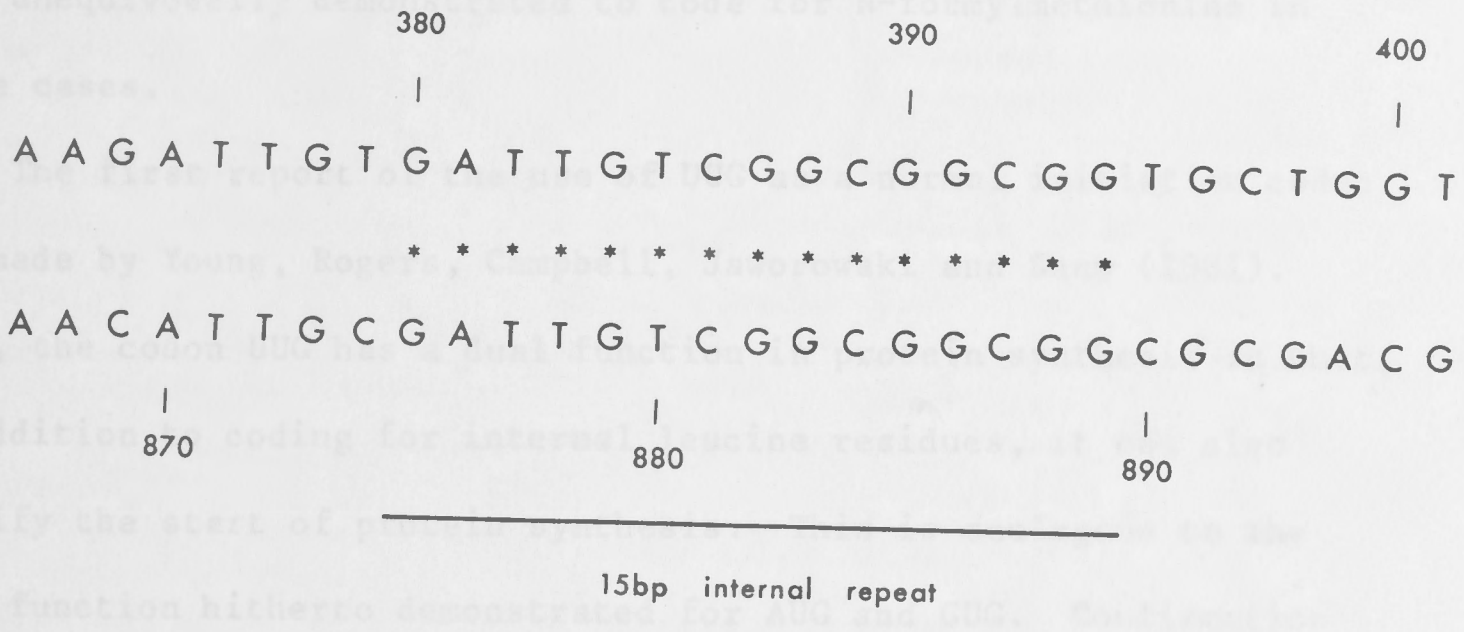
These two regions have subsequently been identified as part of the two nucleotide binding sites possessed by this enzyme and are discussed in a later section of this Chapter.

Initiation Codon Sequences

Early studies by Ghosh, Sȣll and Khorana (1967) established that AUG, GUG and UUG trinucleotides were capable of binding fmet-tRNA to ribosomes. Furthermore, the two forms of E. coli tRNA fmet responded similarly in the ribosome binding assay to the triplets AUG, GUG and UUG, in decreasing order of effectiveness (Cory, Dube, Clark and Marker, 1968; Weiss, Pearson and Kelmers, 1968). Subsequently, AUG has become well established as the usual initiation codon in vivo (Gold, Pribnow, Schneider, Shinedling, Singer and Stormo, 1981).

In the sequence compilation of Gold et al. (1981) four of the 124 translational initiation sequences have GUG rather than the far more prevalent AUG initiator (see also Stormo et al., 1982b). The reason why GUG is occasionally used as an initiation codon is as yet

Figure 2-11. An Internal Sequence Repeat at Nucleotides 380-394 and 875-889 in the Ndh Structural Gene



unexplained. A list of E. coli genes that have been reported to use GUG as an initiation codon is given in Table 2-1. The mRNAs that use GUG as an initiation codon encode proteins that have no obvious similarity. It should be noted that in some of the reports in Table 2-1, protein chemical data was not provided and hence GUG has not been unequivocally demonstrated to code for N-formylmethionine in these cases.

The first report of the use of UUG as a normal initiation codon was made by Young, Rogers, Campbell, Jaworowski and Shaw (1981). Thus, the codon UUG has a dual function in protein synthesis in that, in addition to coding for internal leucine residues, it can also signify the start of protein synthesis. This is analogous to the dual function hitherto demonstrated for AUG and GUG. Confirmation that the initiating codon UUG codes for methionine in the ndh gene has come from studies in which the NADH:ubiquinone oxidoreductase has been synthesized in vitro using a transcription/translation system, under conditions where the post-translational processing is considerably reduced (Poulis et al., 1981). The results indicated that the first residue of the unprocessed NADH dehydrogenase is N-formylmethionine.

Soon after the first report of the use of UUG as a normal initiation codon (Young et al., 1981) two other reports have been published. Mackie (1981) reported that the gene for ribosome protein S20 (rpsT) of E. coli utilized UUG as the initiation codon and McLaughlin, Murray and Rabinowitz (1981) reported that UUG acts as an initiation codon for the Staphylococcus aureus β-lactamase gene. As with some of the reports listed in Table 2-1, the evidence presented by Mackie (1981) for the use of UUG as the initiation codon is an in-

Table 2-1. *E. coli* and Coliphage mRNAs that have been reported to use GUG as the Initiation Codon *in vivo*

Protein	Gene	Reference
coliphage MS2 A-protein	-	Fiers (1979)
lactose repressor	*lacI*	Steege (1977); Farabaugh (1978)
minor capsid protein of bacteriophage FD and M13	geneIII	Beck *et al.* (1978); Wesenbeek and Schoenmakers (1979)
elongation factor Tu	*tufA*	Yokota, Sugisaki, Takanami and Kaziro (1980)
ribosomal protein S13	*rspM*	Post, Arfsten, Davis and Nomura (1980)
β' subunit of RNA polymerase	*rpoC*	Ovchinnikov *et al.* (1981)
coliphage T7 protein 2.8	-	Dunn and Studier (1981)
alkaline phosphatase	*phoA*	Kikuchi, Yoda, Yamasaki and Tamura (1981)
flavoprotein subunit of fumarate reductase	*frdA*	Cole (1982)
protein-b of the F_o component of the ATP-synthase	gene 4 of the *unc* operon	Gay and Walker (1981)
histidyl-tRNA synthetase	*hisS*	Eisenbeis and Parker (1982)
resistence transfer factor	*repA1*	Brawner and Jaskunas (1982)
histidine transport[a] protein	*hisM*	Higgins, Haag, Nikaido, Ardeshir, Garcia and Ames (1982)

[a] Membrane component of the histidine transport operon of *Salmonella typhimurium*.

phase reading frame of the appropriate length preceded by a Shine-Dalgarno ribosome binding sequence and a putative promoter sequence. McLaughlin et al. (1981), using [^{35}S]-methionine, [^{3}H]-leucine and [^{3}H]-lysine coupled with automated amino acid sequence analysis, demonstrated that the UUG initiation codon codes for methionine at the N-terminus of the leader sequence of the β-lactamase.

More recently, AUU has been reported to act as an initiation codon for the initiation factor IF3 (infC gene) of E. coli (Sacerdot, Fayat, Dessen, Springer, Plumbridge, Grunberg-Manago and Blanquet, 1982). The evidence provided for the use of AUU as an initiation codon included an open reading frame of the appropriate length preceded by a ribosome binding site sequence (ibid.). Unfortunately, no protein sequence data was provided to demonstrate that AUU codes for N-formyl methionine.

Hence, AUG, UUG and GUG have all been shown to act as initiation codons in vivo with the only invariable nucleotide base (thus far) being the conserved central U (AUU has also been suggested).

Codon Usage

The accumulation of extensive sequence data has led to the detection of a genetic control mechanism mediated via codon usage. The use of a mRNA sequence database has established that the genetic code is used differently by different species and each type of genome has a particular coding strategy, that is, choices among degenerate bases are consistently similar for all genes therein (Grantham, Gautier and Gouy, 1980a; Grantham, Gautier, Gouy, Mercier and Pave, 1980b). Contrary claims have been made suggesting that the preferential use of degenerate codons is gene specific (Wain-Hobson,

Nussinov, Brown and Sussman, 1981). Nonetheless, in E. coli it has been established that codon usage is markedly different in highly expressed genes compared with genes coding for rare proteins like repressors (Grantham, Gautier, Gouy, Jacobzone and Mercier, 1981; Gouy and Gautier, 1982; Grosjean and Fiers, 1982).

The codon usage and codon composition of the ndh structural gene of E. coli is shown in Table 2-2. The ndh gene is fairly similar to the E. coli genome in its use of the following codons: (a) preference for CUG in coding for leucine (this unexplained preference occurs also in animals as well as in bacteria), (b) CCG is the favoured codon for proline, and (c) the following codons are not favoured: CGG, AGA, AGG, (arg); CCC, CCU, (pro); AUA, (ile). The ndh gene is atypical in that it utilizes UCG (ser) relatively more often than highly expressed E. coli genes (12 versus 0 codons per 1000, respectively; see Gouy and Gautier, 1982).

Gouy and Gautier (1982) have developed an interesting strategy enabling the confirmation of the correlation of codon usage with gene expressivity (rate of synthesis of a protein and the total amount of it made; defined in Grantham et al., 1980b). Gouy and Gautier (1982) have quantified the influence of tRNA availability based on a model of protein synthesis dynamics (P1 index = average number tRNA discriminations per elongation cycle). They also characterized each messenger in their sequence bank (83 E. coli sequences) by the frequencies of the choices between the pyrimidines among codons beginning with AA, AU, UA, UU, CC, CG, GC or GG (called the P2 index). The relationship between highly expressed genes in E. coli as well as the ndh gene (called ECONDH) with these two calculated indices (P1 and P2) is shown in Figure 2-12. The respiratory NADH

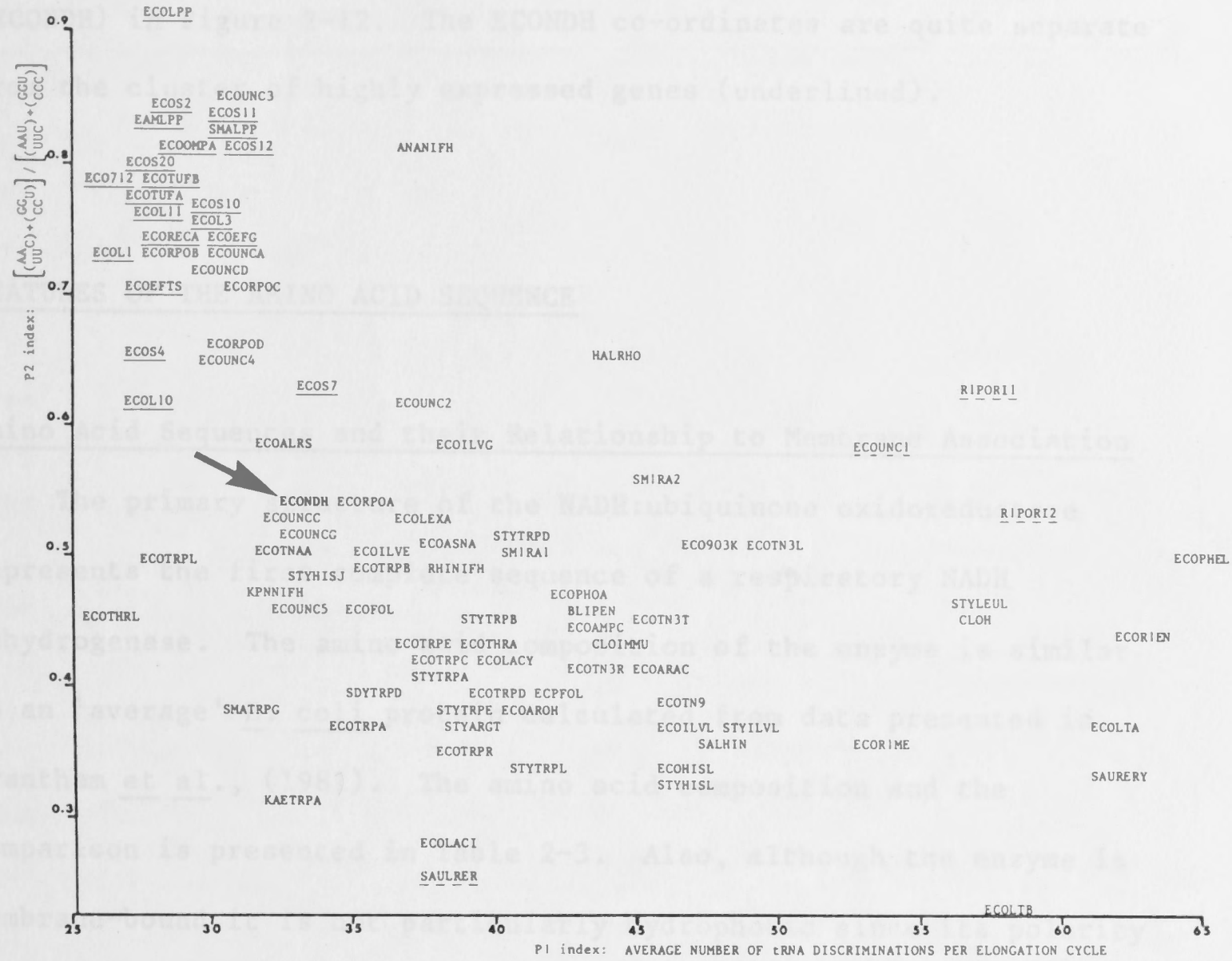
Table 2-2. Tabulation of the Codon Usage and Codon Composition of the Ndh Structural Gene of E. Coli

Amino Acid	Codon	Codon Composition[a]	Codon Usage[b]	Amino Acid	Codon	Codon Composition[a]	Codon Usage[b]
Arg	CGA	5	2	Val	GUA	16	7
	CGC	21	9		GUC	21	9
	CGG	0	0		GUG	16	7
	CGU	25	11		GUU	9	4
	AGA	0	0				
	AGG	0	0	Lys	AAA	44	19
					AAG	14	6
Leu	CUA	5	2				
	CUC	5	2	Asn	AAC	42	18
	CUG	81	35		AAU	12	5
	CUU	7	3				
	UUA	5	2	Gln	CAA	7	3
	UUG	16	8*		CAG	25	11
Ser	UCA	5	2	His	CAC	23	10
	UCC	9	4		CAU	16	7
	UCG	12	5				
	UCU	5	2	Glu	GAA	42	18
	AGC	12	5		GAG	12	5
	AGU	5	2				
				Asp	GAC	12	5
Thr	ACA	5	2		GAU	30	13
	ACC	21	9				
	ACG	21	9	Tyr	UAC	14	6
	ACU	14	6		UAU	14	6
Pro	CCA	12	5	Cys	UGC	9	4
	CCC	0	0		UGU	0	0
	CCG	25	11				
	CCU	2	1	Phe	UUC	18	8
					UUU	7	3
Ala	GCA	12	5				
	GCC	16	7	Ile	AUA	0	0
	GCG	44	19		AUC	18	8
	GCU	18	8		AUU	42	18
Gly	GGA	9	4	Met	AUG	32	14
	GGC	39	17				
	GGG	12	5	Trp	UGG	5	2
	GGU	37	16				

[a] Codon compositions are expressed per 1000 and both initiator and stop codons are excluded from the data (Taken from Gouy and Gautier, 1982).

[b] Codon usage is expressed as the number of codons represented in the ndh structural gene (434 codons). The table lists the atypical initiator UUG codon as encoding Leu not Met (*).

Figure 2-12. Characterization of Bacterial Gene Codon Usage by Number of Non-Specific tRNA Discriminations During Translation and Pyrimidine use in Codon Position III



Genes for the most abundant proteins are underlined and the ndh gene is arrowed. Symbols with a dashed underline are hypothetical genes. The P1 and P2 indices are defined in the text and are taken from Gouy and Gautier (1982).

dehydrogenase is a rather minor component of the cytoplasmic membrane of E. coli and this is reflected in the co-ordinates of the ndh gene (ECONDH) in Figure 2-12. The ECONDH co-ordinates are quite separate from the cluster of highly expressed genes (underlined).

FEATURES OF THE AMINO ACID SEQUENCE

Amino Acid Sequences and their Relationship to Membrane Association

The primary structure of the NADH:ubiquinone oxidoreductase represents the first complete sequence of a respiratory NADH dehydrogenase. The amino acid composition of the enzyme is similar to an 'average' E. coli protein calculated from data presented in Grantham et al., (1981). The amino acid composition and the comparison is presented in Table 2-3. Also, although the enzyme is membrane-bound it is not particularly hydrophobic since its polarity (43.4%) is not unusually low (Capaldi and Vanderkooi, 1972).

The average hydrophobicity index of the NADH dehydrogenase is fairly low (1.95 kJ/mole residue) (calculated according to the method described by Heijne (1980), with modifications for the tyrosine and cysteine residues as recommended by Gilson, Higgins, Hofnung, Ames and Nikaido (1982a)). A comparison between the average hydrophobicity index of the NADH dehydrogenase (not including N-terminal methionine) and some other proteins from E. coli and Salmonella typhimurium is presented in Figure 2-13. This figure is adapted from one presented in Gilson et al. (1982a) where the references and proteins are defined. In Figure 2-13 it is clear that the NADH dehydrogenase (ndh) has a low average hydrophobicity index

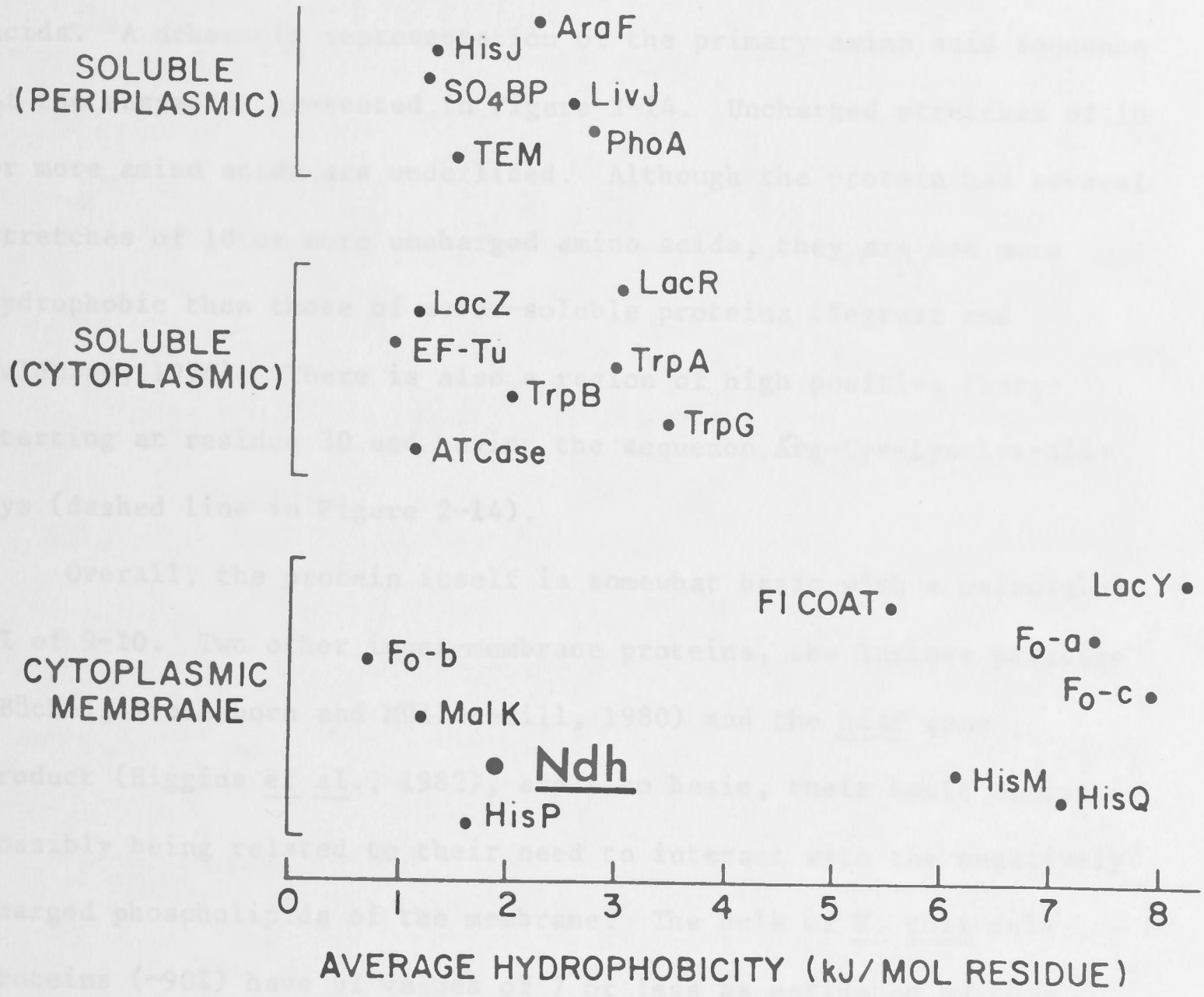
Table 2-3. Amino Acid Compositions of the Ndh Gene Product and 'Average' E. coli Proteins

Amino Acid	Amino Acid Composition[a]	Mol % in: Ndh[a]	Mol % in: Average (E. coli)
Asp	18	4.2	5.2
Asn	23	5.3	3.5
Thr	26	6.0	5.8
Ser	20	4.6	6.3
Glu	23	5.3	5.5
Gln	14	3.2	4.2
Pro	17	3.9	3.5
Gly	42	9.7	7.2
Ala	39	9.0	11.1
Val	27	6.2	7.5
Met	14	3.2	2.2
Ile	26	6.0	6.1
Leu	51	11.8	7.9
Tyr	12	2.8	2.6
Phe	11	2.5	3.6
Lys	25	5.8	6.4
His	17	3.9	2.5
Arg	22	5.0	6.6
Cys	4	0.9	1.1
Trp	2	0.5	1.2

[a] Calculated from sequence and does not include N-terminal Met.

[b] Calculated from data presented in Grantham et al., (1981).

Figure 2-13. Average Hydrophobicity Index of Some Proteins from *E. coli* and *S. Typhimurium*: Comparison with the Hydrophobicity Index of NADH Dehdyrogenase (Ndh)



See text for details. This figure is adapted from one presented in Gilson *et al*. (1982a) where the references and proteins are defined.

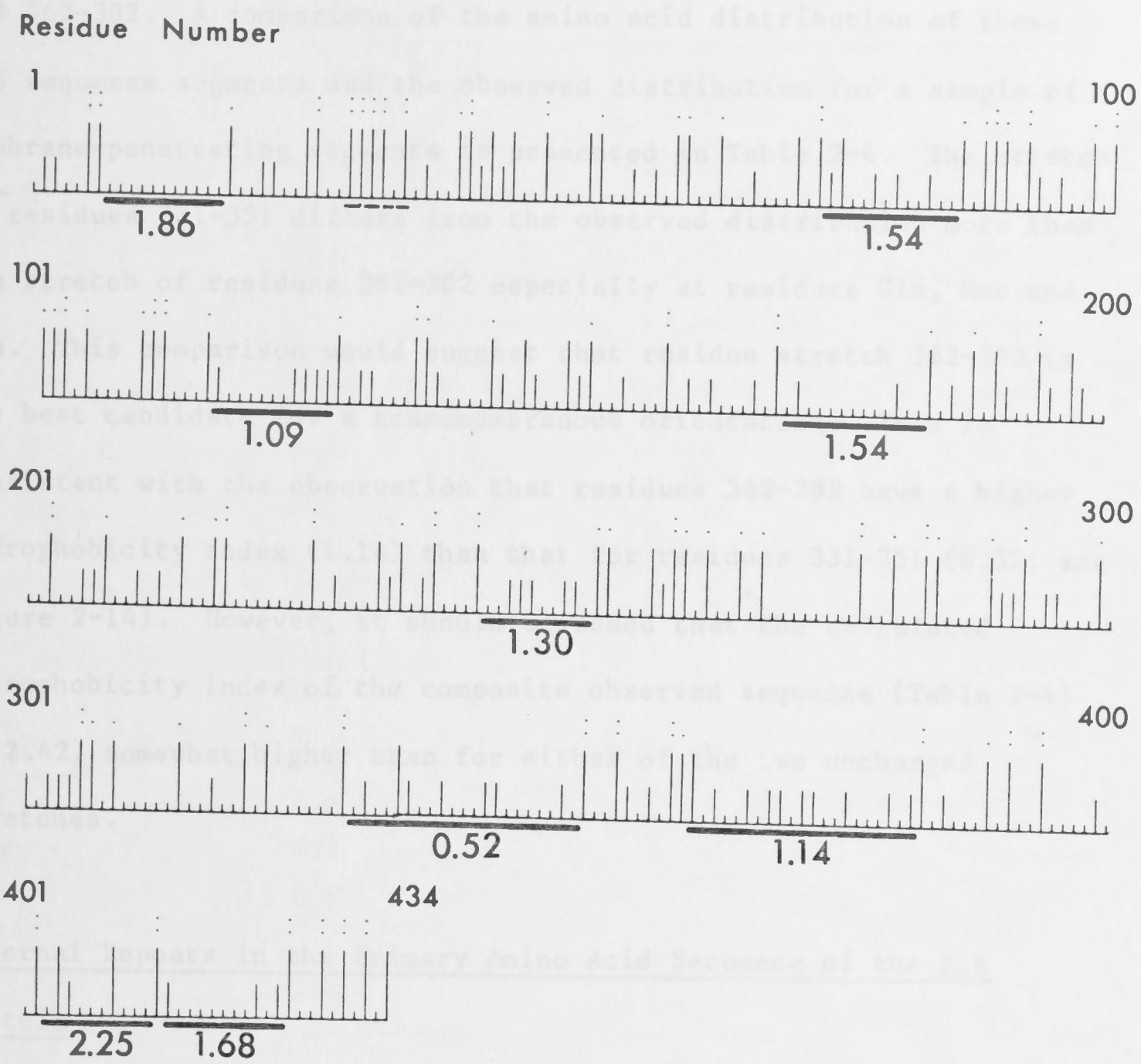
among the inner-membrane proteins and is similar to those of the more hydrophobic members of soluble proteins of E. coli and S. typhimurium.

The enzyme possesses a number of stretches of uncharged amino acids. A schematic representation of the primary amino acid sequence of the enzyme is presented in Figure 2-14. Uncharged stretches of 10 or more amino acids are underlined. Although the protein has several stretches of 10 or more uncharged amino acids, they are not more hydrophobic than those of water-soluble proteins (Segrest and Feldmann, 1974). There is also a region of high positive charge starting at residue 30 and having the sequence Arg-Lys-Lys-Lys-Ala-Lys (dashed line in Figure 2-14).

Overall, the protein itself is somewhat basic with a calculated pI of 9-10. Two other inner-membrane proteins, the lactose permease (Büchel, Gronenborn and Müller-Hill, 1980) and the hisP gene product (Higgins et al., 1982), are also basic, their basic character possibly being related to their need to interact with the negatively charged phospholipids of the membrane. The bulk of E. coli cell proteins (~90%) have pI values of 7 or less as estimated by two-dimensional gel electrophoresis of total cell protein (O'Farrell, 1975).

In a list of the membrane-penetrating segments compiled by Heijne (1981), the length of each membrane-associated segment was approximately 20 residues of which none possessed a charged residue (arg, lys, glu or asp) or a pro residue. Using these two criteria, a length of at least 20 consecutive uncharged amino acids and the absence of a pro residue (a strong helix breaker; Chou and Fasman, 1978) there are only two possible candidates for transmembrane segments in the NADH dehydrogenase sequence. These are the two

Figure 2-14. Schematic Representation of the Primary Amino Acid Sequence of the Ndh Gene



Each amino acid in the ndh sequence is represented as a bar. The different residues are represented in the one letter amino acid abbreviations as follows: small bar, M,P,L,I,V,G,A,F,Y,C and W; half bar, Q,T,S,N; full bar, H; full bar with two dots, K,R and full bar with one dot, D,E. Stretches of 10 or more uncharged amino acids are underlined and a region of high positive charge starting at residue 30 is indicated by the dashed line. The hydrophobic index (Segrest and Feldmann, 1974) is listed underneath each uncharged sequence stretch.

uncharged 21 amino acid stretches spanning residue numbers 331-351 and 362-382. A comparison of the amino acid distribution of these two sequence segments and the observed distribution for a sample of membrane-penetrating segments is presented in Table 2-4. The stretch of residues 331-351 differs from the observed distribution more than the stretch of residues 362-382 especially at residues Gln, Met and Asn. This comparison would suggest that residue stretch 362-382 is the best candidate for a transmembranous orientation. This is consistent with the observation that residues 362-382 have a higher hydrophobicity index (1.14) than that for residues 331-351 (0.52; see Figure 2-14). However, it should be added that the calculated hydrophobicity index of the composite observed sequence (Table 2-4) is 2.42, somewhat higher than for either of the two uncharged stretches.

Internal Repeats in the Primary Amino Acid Sequence of the Ndh Protein

The detection of an obvious 15 bp internal repeat in the ndh structural gene (Figure 2-11) gave impetus to a more thorough search for further internal homology. Using visual inspection (Dr H.D. Campbell) and the Staden (1977) program SEQFIT two large repeats of approximately 130 amino acids in the first ~300 residues and two smaller repeats of ~20 amino acids near the C-terminus were detected (Campbell, Rogers and Young, 1982). The probabilities of these repeats arising by chance, as assessed by the computer program ALIGN (Dayhoff, 1978) are $<10^{-15}$ and 10^{-10}, respectively (Campbell, Rogers, Young, Yeh and Dayhoff, 1983). These findings are indicative of a primordial gene duplication. The segments with the best homologies are listed in Figure 2-15.

Table 2-4. Observed Amino Acid Distribution for a Sample of Membrane-Penetrating Segments: Comparison with Two Long (21 residues) Uncharged Amino Acid Stretches in the Ndh Sequence

Amino Acid	Observed[a]	Number of Residues 331-351[b]	362-383[b]
Leu	3.9	1	4
Ala	2.3	6	0
Val	2.2	0	2
Phe	1.8	0	1
Ser	1.8	0	5
Ile	2.5	1	0
Pro	0	0	0
Gly	1.5	1	3
Cys	1.2	1	0
Met	0.9	3	1
Thr	1.3	1	2
Tyr	0.4	0	0
Gln	0.1	3	0
Trp	0.1	0	0
Asn	0	3	2
His	0	1	1
Total	20.0	21	21

[a] Number of residues/20 amino acids; derived from a composite of 10 known membrane-penetrating sequences (Heijne, 1981)

[b] Residue numbering taken from sequence.

Figure 2-15. Regions of Internal Sequence Homology in the E. coli Respiratory NADH Dehydrogenase

Regions compared[a]		
8- 24[b]	A :	I V I V G G G A G G L E M A T Q L
		* * * * * * * * * *
173-189	A':	I A I V G G G A T G V E L S A E L
39- 61	B :	V D R N H S H L W K P L L H E V A T G S L D E
		* * * * * * * * * * *
193-215	B':	V K Q L H S Y G Y K G L T N E A L N V T L V E
65- 82	C :	A L S Y L A H A R N H G F Q F Q L G
		* * * * * * * *
223-240	C':	A L P P R I S A A A H N E L T K L G
93-129	D :	T I T I A E L R D E K G E L L V P E R K I A Y D T L V M A L G S T S N D F
		* * * * * * * * * * * * * * * * * * *
245-281	D':	T Q T M V T S A D E G G L H T K D Y E Y I E A D L M V W A A G I K A P D F
133-139	E:	G V K E N C I
		* * * *
287-293	E':	G L E T N R I
377-394	F :	L M G N L T R G S M M I E G R I A R
		* * * * * * * * * * *
408-425	F':	L H G Y F K T G L M M L V G S I N R

[a] Residue numbering is taken from sequence (see Figure 2-7).

[b] Identical residues are indicated by an asterisk.

Sequence Homology Between the NADH Dehydrogenase and Other Enzymes

Evolutionary relationships between dehydrogenases (Rossman, Liljas, Brändén and Banaszak, 1975; Jörnvall, 1980) and pyridine nucleotide disulphide oxidoreductases (Holmgren, 1980) have been subjected to scrutiny in recent years.

With the aim of elucidating the evolutionary relatedness of the NADH dehydrogenase to other proteins a small protein sequence database was compiled and protein sequences were subjected to sequence comparisons using the program SEQFIT (Staden, 1977). Special attention was given to oxidoreductases and respiratory enzymes. A number of proteins were found to have some sequence homology with the E. coli respiratory NADH dehydrogenase.

One enzyme which was shown to possess homology with the NADH dehydrogenase was the human erythrocyte glutathione reductase (Krauth-Siegel, Blatterspiel, Saleh, Schiltz, Schirmer and Untucht-Grau, 1982). The glutathione reductase subunit (mol wt 50000) consists of three structural domains corresponding to binding of FAD and NADPH and a flat interface domain where glutathione disulphide is bound (Holmgren, 1980; Schulz, Schirmer, Sachsenheimer and Pai, 1978). The two nucleotide-binding domains of the flavo-enzyme have similar chain folds and are believed to have arisen by gene duplication (Schulz, 1980). The enzyme is well characterized and its three dimensional structure (Thieme, Pai, Schirmer and Schulz, 1981) and complete sequence is known (Krauth-Siegel et al., 1982; Untucht-Grau, Schirmer, Schirmer and Krauth-Siegel, 1981). Amino acid sequence homology studies suggest that lipoamide dehydrogenase, thioredoxin and glutathione reductase are all evolutionarily related (Holmgren, 1980). More recently, it has been

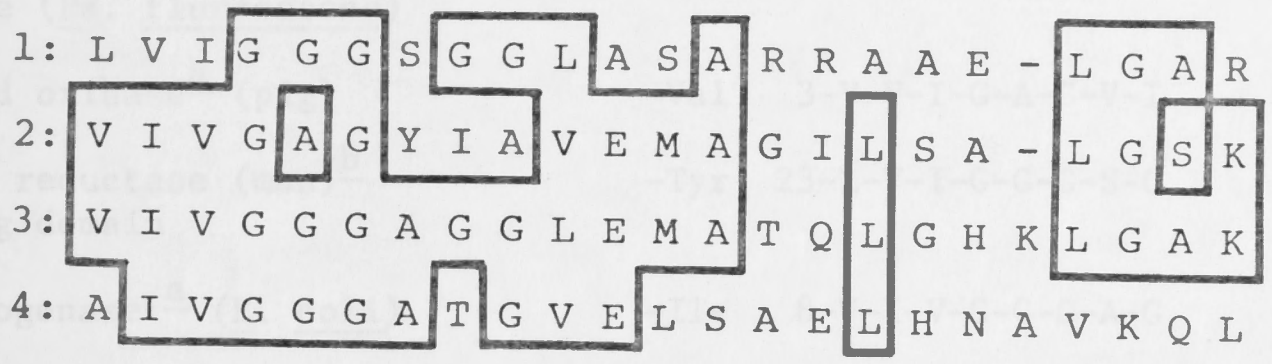
shown that the chain-fold of the FAD-binding domain of p-hydroxybenzoate hydroxylase resembles the chain folds of the two nucleotide-binding domains of glutathione reductase (Wierenga, Drenth and Schulz, 1983).

Upon comparing the sequences of the E. coli NADH dehydrogenase and the human erythrocyte glutathione reductase, a striking section of sequence homology between the duplicated regions was observed (Figure 2-16). The probability of the sequence homology being due to chance, as assessed by the computer program ALIGN (Dayhoff, 1978) is $<10^{-9}$ (Campbell et al., 1983).

The internal sequence homologies within the NADH dehydrogenase, by analogy with the two known glutathione reductase domains, allow the assignment of a particular function to each of the repeated internal sequence homologies. Consequently, it is concluded that the internal sequence repeats (regions defined as A, B, C, D and E, and A', B', C', D' and E' in Figure 2-15) can be assigned as the FAD and NADH nucleotide binding domains, respectively. The NADH dehydrogenase sequence is compared with the known sequence and correlated with the known three-dimensional structure of glutathione reductase in Table 2-5.

It should not be implied from this data that all pyridine nucleotide binding domains are homologous. For example, structural studies suggest that all of the following enzymes have NADPH-binding domains that are different from one another in their chain folds and are not homologous: L. casei dihydrofolate reductase, spinach ferredoxin reductase, Ps. fluoescens p-hydroxybenzoate hydroxylase and liver 6-phosphogluconate dehydrogenase (Krauth-Siegal et al., 1982).

Figure 2-16. Sequence Homology between Duplicate Regions in the Human Glutathione Reductase and the E. coli Respiratory NADH Dehdyrogenase



1: Glutathione Reductase,residues 24-45

2: Glutathione Reductase,residues 191-212

3: NADH Dehydrogenase,residues 9-31

4: NADH Dehydrogenase,residues 174-196

The residue numbering is as described in Krauth-Siegel et al. (1982) and Figure 2-7, respectively.

Table 2-5. The N-terminal β-Sheet Strand and the Subsequent Pyrophosphate-Binding Loop in FAD-Dependent Enzymes and in Flavodoxin

Protein[c]		Sheet strand	Phosphate loop
p-Hydroxybenzoate hydroxylase (Ps. fluroescens)	-Val	5	-A-I-I-G-A-G-P-S
D-amino acid oxidase[a] (pig)	-Val	3	-V-V-I-G-A-G-V-I
Glutathione reductase (man)[b] FAD-binding domain	-Tyr	23	-L-V-I-G-G-G-S-G
NADH dehydrogenase [a] (E. coli)	-Ile	8	-V-I-V-G-G-G-A-G
Flavodoxin (Clostridium MP)	-Val	4	-Y-W-S-G-T-G-N-T
Adenylate kinase (pig, man)[d]	-Ile	11	-F-V-V-G-G-P-G-S
Lipoamide dehydrogenase[a] (pig)	-Val	9	-X-V-I-G-S-G-P-G
Glutathione reductase[d] NADPH-binding domain (man)	-Ser	190	-V-I-V-G-A-G-Y-I
NADH dehydrogenase[a,d] (E. coli)	-Ile	173	-A-I-V-G-G-G-A-T

[a] Tertiary structures are unknown; for these flavoenzymes the alignment is based on sequence.

[b] As indicated by the position numbers (Val-5, Val-3, etc), the strand sheet which is located in the protein's interior is very close to the N-terminus. The special case of the long N-terminal extension is discussed in Untucht-Grau et al., (1981).

[c] References defined in Untucht-Grau et al., (1981).

[d] Adenylate kinase and the NADPH-domain of glutathione reductase which do not bind FAD nor FMN represent numerous other nucleotide binding structures. NADH dehydrogenase binds NADH not NADPH.

Interestingly, the frdA gene product sequence and the beef heart succinate dehydrogenase share nine consecutive residues at their flavin binding site (Cole, 1982). The FAD cofactor is believed to be covalently bound to a histidyl residue (residue 45 in the frdA protein, Cole, 1982; Weiner and Dickie, 1979). The NADH dehydrogenase also has a histidine at position 45, however there is no discernible sequence homology in the vicinity of the histidyl residue.

Another member of the disulphide reductase class of flavoproteins, mercuric reductase, which possesses striking spectroscopic similarities to the lipoamide dehydrogenase and glutathione reductase has recently been purified and characterized (Fox and Walsh, 1982). In contrast to the three disulphide reductases, mercuric reductase catalyzes no net reduction of a substrate disulphide but rather the more unusual two-electron reduction of inorganic Hg^{2+}. It will be interesting to see when sequence data becomes available, whether this enzyme shares homology with the NADH dehydrogenase.

The two smaller repeats of approximately 20 amino acids near the C-terminus (probabilities of these repeats arising by chance $<10^{-10}$; Campbell et al., 1983) are not related to any section of sequence from glutathione reductase. The function of the short C-terminal repeats (assigned as F/F' in Figure 2-15) is unknown. One possibility is that they are involved in ubiquinone reduction. If this is so, the ubiquinone 'domain' may have come about by a gene fusion event as has occurred in the evolution of other dehydrogenases (Rossman et al., 1975).

The complete nucleotide sequence of the malK gene product of E. coli has recently been published (Gilson, Nikaido and Hofnung, (1982b). Gilson et al., (1982b) claim to have found significant homology between the sequence of the malK protein and that of the respiratory NADH dehydrogenase of E. coli. However, the homology itself is marginal and Gilson et al. (1982b) admitted difficulty in assigning any functional significance to the putative homology.

SUMMARY

The complete nucleotide sequence for the ndh structural gene has been determined using the Sanger chain-termination method (Sanger, 1981). This represents the first respiratory dehydrogenase to be sequenced (Young et al., 1981). Analysis of the sequence has revealed the following features: (a) A putative promoter approximately 40 bp upstream from the initiation codon, (b) two inverted repeats, one upstream from the initiation codon (covering sections of the putative promoter), and the other immediately after the stop codon. The latter repeat is believed to act as an RNA transcription termination structure. (c) A ribosome binding site, complementary to the 16S ribosomal RNA immediately precedes the initiation codon. (d) UUG, which codes for N-formylmethionine rather than the usual leucine residue, acts as an atypical initiation codon.

The ndh structural gene codon composition and codon usage is consistent with the NADH dehydrogenase being only a minor component of the total E. coli protein.

The amino acid composition of the NADH dehydrogenase is indistinguishable from an 'average' composite E. coli protein deduced from a sequence database. The enzyme is not particularly hydrophobic as the following parameters indicate: polarity = 43.4% (Capaldi and Vanderkooi, 1972) and the average hydrophobicity index = 1.95 kJ/mole residue as calculated according to Heijne (1980) and Gilson et al., (1982a).

There are nine stretches of ten or more uncharged residues in the NADH dehydrogenase amino acid sequence. The hydrophobic stretches tend to be more prevalent in the last 100 residues of the enzyme. Calculation of the hydrophobicity indices (Segrest and Feldmann, 1974) of the uncharged stretches showed that none were distinguishable from uncharged stretches present in known soluble proteins. Using sequence comparisons between a composite membrane-penetrating sequence (Heijne, 1981) it was deduced that only one amino acid stretch (residues 362-382) is likely to be transmembranous.

An examination of the primary amino acid sequence, both by manual inspection (Dr H.D. Campbell) and the use of the computer program SEQFIT (Staden, 1977) led to the detection of internal homology within the enzyme. There are two large repeating units of ~130 amino acids near the N-terminus of the protein and two smaller repeats of ~20 amino acids near the C-terminus. The probabilities of these repeats arising by chance, as assessed by the computer programme ALIGN, are 10^{-15} and 10^{-10}, respectively (Campbell et al., 1983).

Comparison of the primary amino acid sequence of the enzyme with other proteins led to the detection of homology with glutathione

reductase ($P<10^{-9}$). By comparison with the well known three-dimensional structure of glutathione reductase, the two large N-terminal repeats of the NADH dehydrogenase can be assigned as the FAD and NADH binding domains. The function of the short C-terminal repeats is unknown. One possibility is that they are involved in ubiquinone reduction.

Chapter 3

Catalytic Activity of Purified and Reconstituted *E. coli* NADH Dehydrogenase with different Ubiquinone Homologues

INTRODUCTION

The Role of Ubiquinone in Electron Transport

Ubiquinone was first postulated to be a member of the electron transport chain primarily because this ubiquitous, neutral lipid is capable of oxidation-reduction and is present in mitochondria (Crane, Hatefi, Lester and Widmer, 1957). Some twenty years after this proposal, ubiquinone is now well established as an obligatory redox component of the electron transport chain of mitochondria and some bacteria (Trumpower and Katki, 1979). Some of the most compelling evidence for its obligatory role comes from extraction and reactivation studies in mitochondria (Lester and Fleischer, 1959; Ernster *et al.*, 1969a,b) and studies with ubiquinone-deficient mutants of *E. coli* (Cox *et al.*, 1970; Wallace and Young, 1977a,b) and *Saccharomyces cerevisiae* (De Kok and Slater, 1975). The mechanism of ubiquinone function and the number of sites at which it acts, however, remains less well understood.

The mobile carrier hypothesis states that there is a single, homogeneous pool of ubiquinone which diffuses between the primary electron transport complexes in the phospholipid continuum of the membrane and that there are specific sites for oxidation-reduction of ubiquinone located in the lipoprotein domains of the dehydrogenase complexes and the bc_1 complex (in the case of mitochondria). Kröger and Klingenberg (1970;1973a,b) have investigated the mobile carrier hypothesis and have shown that the kinetics of oxidation-reduction and the steady state redox characteristics of ubiquinone are consistent with a model in which 80-90% of the ubiquinone exists as a functionally homogeneous pool and there are not separate

compartments of ubiquinone for the different substrates such as NADH and succinate.

More recently, Zhu, Berden, De Vries and Slater (1982) have suggested that free mobile ubiquinone is not necessarily required for apparent pool-function behaviour and that both direct interaction between ubiquinone-loaded enzymes and diffusion of ubiquinone and ubiquinol are involved in the electron transfer from the dehydrogenases to the ubiquinol:cytochrome c oxidoreductase.

Studies involving electron paramagnetic resonance (EPR) spectrometry have indicated the existence of ubisemiquinones in the electron transport chain of mitochondria (Ingledew, Salerno and Ohnishi, 1976; Ruzicka, Beinert, Schepler, Dunham and Sands, 1975). As pointed out by Ingledew et al. (1976) the ubisemiquinones must be located in an ubiquinone-protein complex and could not be free to diffuse because this would lead to disproportionation and subsequent loss of EPR signals. However, the percentage of ubiquinone which is present as ubisemiquinone is calculated to be very small in the mitochondrial membrane (≤1%; Crane, 1977) and in the inner-membrane of E. coli (<2%; Cox et al., 1970). The role of ubisemiquinone in electron transfer is still uncertain.

Hence, the view that the bulk of the ubiquinone in the membrane functions as a mobile carrier of reducing equivalents is not universally accepted. It has also been proposed that the actual carrier is ubiquinone bound to specific ubiquinone binding proteins (QPs; M_r 12000-15000; Yu, Yu and King, 1977; Yu and Yu, 1980a). Furthermore, it has been asserted that there is a ubiquinone binding protein functioning in the mitochondrial NADH:ubiquinone oxidoreductase (Yu and Yu, 1981). However, to date the most pure QP preparation contains at least 10% impurities including detectable

cytochrome b (Yu and Yu, 1981). Also, Hatefi and Galante (1980) demonstrated that purified cytochrome b_{560} (two peptides of M_r 13500 and 15000) of Complex II is able to reconstitute the succinate: ubiquinone reductase complex when added to purified succinate dehydrogenase. It is not clear at present whether the QPs isolated by Yu et al. (1977) and the cytochrome b_{560} preparation of Hatefi and Galante (1980) are related.

Recent work by Schneider et al. (1980;1982) has provided further experimental evidence to support the earlier proposals by Green and Wharton (1963) and Krȍger and Klingenberg (1973a,b) that ubiquinone is a mobile electron carrier which functions as a homogeneous pool during the transfer of electrons between dehydrogenases and the cytochrome bc_1 complex. Schneider et al. (1980) examined the rates of specific catalytic events in the mitochondrial inner membrane after enriching the membrane bilayer with exogenous phospholipid. From this data, they concluded that a diffusion-limited step was involved in the transfer of reducing equivalents from the dehydrogenases to cytochromes bc_1 in the native membrane. Similar experiments by Schneider et al. (1982) gave results which did not support proposals in which ubiquinone functions as a protein-bound electron carrier (for further discussion see Chapter 1).

The chemiosmotic hypothesis formulated by Mitchell (1961;1973) has greatly contributed to our knowledge of the role of the 'protonmotive force' in bioenergetics and the nature of the electrochemical proton gradient. The proton-motive ubiquinone cycle, proposed by Mitchell (1975a,b;1976) suggested a direct role for ubiquinone in the generation of the electrochemical proton gradient. The view that lipophilic quinones can be used to establish transmembrane proton gradients is supported by studies of

nonenzymatic ubiquinone oxidation in a model lipid-bilayer system, which showed that autooxidation of ubiquinol-6 formed by addition of NADH on one side of the lipid-bilayer caused the formation of a proton gradient across the membrane (Yaguzhinsky, Boguslavsky and Ismailov, 1974).

The Role of Ubiquinone in E. coli

Studies by Cox et al. (1970) on the role of ubiquinone in E. coli detected the existence of ubisemiquinone by EPR spectrometry. They proposed a scheme in which ubisemiquinone, complexed to an electron carrier, functions in the electron-transport sequence.

Further studies by Downie and Cox (1978) on the amounts of cytochromes reduced during aerobic steady-state conditions suggested that ubiquinone functions at two sites, one site being between the dehydrogenases and cytochromes and the second site being after cytochromes b_{562} and b_{556} but before cytochromes b_{558}, d and o. However, due to difficulties involved in the measurement of the amount of cytochrome b_{562} reduced, the exact position of the first ubiquinone site is in doubt (Downie and Cox, 1978).

Wallace and Young (1977b) used a double quinone mutant of E. coli, ubiA menA, to examine the role of quinones in electron transport to oxygen and nitrate. They demonstrated that each of the four oxidases examined (NADH, D-lactate, α-glycerophosphate and succinate) requires a quinone for activity. Furthermore, ubiquinone is active in each oxidase system while menaquinone gives full activity in α-glycerophosphate oxidase, partial activity in D-lactate oxidase but is inactive in NADH and succinate oxidation (Wallace and Young, 1977b; see electron transport scheme in Chapter 1).

Wallace and Young (1977b) concluded that the quinones serve to link the various dehydrogenases with the terminal electron transport systems to oxygen and nitrate and that the dehydrogenases possess a degree of selectivity with respect to the quinone acceptors. However, Wallace & Young, (1977a,b) did not exclude the possibility that ubiquinone functions at other sites in the respiratory chain.

Experiments with other ubiquinone mutants of E. coli (ubiE, ubiF and ubiG mutants) indicated that certain substituents of the benzoquinone ring of ubiquinone-8 are important in determining whether or not the quinone can interact with each of the various dehydrogenases involved in electron transport (Wallace and Young, 1977a). These results provided further support for the concept that each dehydrogenase has specific structural requirements for quinone acceptors. In contrast to the ring substituents, the length of the isoprenoid side chain is a less critical feature of the ubiquinone-8 molecule for activity in the four oxidases tested. As shown by Wallace and Young (1977b), when ubiquinone-3 was added to ubiA menA membranes the rates of oxidation of NADH, D-lactate, α-glycerophosphate and succinate were approximately equal to those found in men membranes. Moreover, electron transport promoted by ubiquinone-3 was found to be coupled to phosphorylation.

Inhibitors of Electron Transfer Mediated by Ubiquinone

Inhibitors have played a vital role in the elucidation of the function of various components of the respiratory chain. Inhibitors have especially contributed to the detection of artifactual activities, such as the ubiquinone reductase of the type II mitochondrial NADH dehydrogenase. Hatefi and Stiggall (1976) pointed out that not only does the type I and type II NADH dehydrogenase

preparations differ in molecular weight, substrate reductase activities and iron-sulphur content but piericidin A, which is the most potent inhibitor known for ubiquinone reduction by complex I and submitochondrial particles, is ineffective on ubiquinone reduction by the soluble enzyme (type II dehydrogenase). Furthermore, substantial inhibition of NADH:ubiquinone-1 reductase activity of the soluble, low molecular weight enzyme requires more than 100-fold as much rotenone as is necessary for a comparable degree of inhibition of complex I. Both rotenone and piericidin are believed to act at the same site (Ragan, 1976a). It is interesting that the $Fe(CN)_6^{3-}$ reductase of the type I dehydrogenase (Singer et al., 1973) and complex I (Hatefi and Stiggall, 1976) is not appreciably inhibited by rotenone.

Schatz and Racker (1966) described experiments involving various electron acceptors that suggest that oxidative phosphorylation at the first phosphorylation site is only observed if electrons passed through the rotenone-sensitive site in submitochrondrial particles. In the same report, a rotenone-insensitive 'group' in the NADH dehydrogenase which reduces ubiquinone-1 was described (Schatz and Racker, 1966). Singer et al. (1973) also noted that in experiments with different electron transport particle preparations, inhibition by rotenone and piericidin was 'leaky', a small fraction of NADH oxidase remains. Wan, Williams and Folkers (1975) corroborated these reports when they demonstrated that 5-7% of the NADH:ubiquinone-1 oxidoreductase activity of electron transport particles still remained in the presence of rotenone (2 μM concentration).

Inhibitors of the electron transport chain of E. coli have also been reported. Early studies by Jones (1967) verified that the NADH oxidase activity of E. coli W was inhibited (~85%) by HQNO at a

concentration of 60 μM. Bragg and Hou (1967a) also found that the NADH oxidase of E. coli was sensitive to inhibition by HQNO (62% and 81% inhibition with HQNO at concentrations of 16 μM and 32 μM, respectively). Cox et al. (1970) found that the extent of HQNO inhibition of the E. coli NADH oxidase was similar to that previously reported by Jones (1967), and Bragg and Hou (1967a) but they also demonstrated that the inhibition was reversed by the addition of ubiquinone-1.

Ubiquinone Reductase Activity of Purified E. coli NADH Dehydrogenase

Many experimental approaches have been used in attempts to elucidate the role of ubiquinone in electron transfer. One major shortfall is that these experiments have generally been performed on very complex preparations composed of many proteins, a large number of which are poorly characterized. The purification of the E. coli respiratory NADH dehydrogenase, which is composed of a single polypeptide (Jaworowski et al., 1981a) of known amino acid sequence (Young et al., 1981) provides a unique opportunity to investigate the properties of ubiquinone reduction by one polypeptide uncomplicated by the complexity of an entire respiratory chain.

Ubiquinone-8 has been established as the in vivo substrate for the E. coli respiratory NADH dehydrogenase (Wallace and Young, 1977a,b). However, the solubility of this compound in aqueous solution is low and this led to the rejection of ubiquinone-8 as a substrate for routine use.

Previous work in this laboratory on the solubilization and purification of the E. coli NADH dehydrogenase used ubiquinone-1, a water-soluble short chain homologue of ubiquinone, as acceptor (Jaworowski et al., 1981a). Ubiquinone-1 can reconstitute NADH

oxidase activity in ubi men membrane vesicles (Cox et al., 1970; Young, unpublished results) and can act as a soluble substrate for both purified enzyme and native E. coli membrane vesicles (Jaworowski et al., 1981a).

Since for routine assays ubiquinone-1 is employed as substrate, whereas the in vivo substrate is ubiquinone-8, verification that the purified enzyme is capable of reducing long chain isoprenoid ubiquinones is required.

In the present study the catalytic activity of the purified E. coli NADH dehydrogenase has been examined with short and long chain homologues of ubiquinone. The purified enzyme has been reconstituted into soybean phospholipid (neutral lipid free) vesicles and alterations in the biochemical properties of the enzyme have been examined.

MATERIALS AND METHODS

Chemicals

Ubiquinone-1 was kindly supplied by Dr O. Isler (F. Hoffman-La Roche and Co. Basel, Switzerland). NADH was from P-L Biochemicals (Milwaukee, WI), and FAD was from Sigma Chemical Co. (St. Louis, MO). N-Tris (hydroxymethyl) methyl-2-aminoethanesulphonic acid, sodium salt (Tes), was obtained from Calbiochem (La Jolla, CA). 2-n-heptyl-4-hydroxyquinoline N-oxide (HQNO) was obtained from Sigma Chemical Company (St. Louis, MO). Purified cholic acid was kindly supplied by Dr H.D. Campbell and was twice recrystallized from 50% (v/v) aqueous ethanol by a procedure similar to that of Hatefi (1978). Stock 20% (w/v) solutions of potassium cholate, pH 7.5, were

prepared by suspending 20 g of cholic acid in ~80 mL of H_2O and titrating the mixture to pH 7.5 over several hours with concentrated KOH. The completely colourless solution was then made up to 100 mL, filtered, and stored at room temperature. $K_3Fe(CN)_6$ used for NADH: $Fe(CN)_6^{3-}$ oxidoreductase activity measurements was of analytical reagent grade (BDH (Australia) Pty Ltd, Sydney). KCN used as an inhibitor of the terminal oxidase of the respiratory chain was analytical reagent grade (BDH (Australia) Pty Ltd, Sydney). All organic solvents were of analytical reagent grade.

Preparation of Ubiquinone-8

Up to 30 g (wet weight) of cells were placed into an extraction thimble and then extracted with acetone (150 mL) in a Soxhlet extraction apparatus for 4 hours. The acetone extract was evaporated to dryness on a steam bath, the residue extracted three times with 50 mL light petroleum (b.p. 60-80°C) and the extract concentrated. The combined extract was chromatographed on a GF_{254} (Merck) silica gel plate (0.5 mm thick, 18 x 20 cm). The chromatograms were developed in $CHCl_3$:petroleum spirit (b.p. 60-80°C), 7:3 (v/v) and the dark yellow ubiquinone band (R_f = 0.3), was removed from the silica plate and eluted six times with 2 mL redistilled absolute ethanol (Merck, AnalR).

The ubiquinone-8 concentration was determined spectrophotometrically by measuring the difference in absorbance at 275 nm after reduction (5 minutes) with solid $NaBH_4$ (Sigma, Analytical Reagent) and using $\Delta\varepsilon$ = 12500 M^{-1} cm^{-1} (Crane and Barr, 1971).

Assays

Routine assays were performed using a Varian 634 series spectrophotometer. Ultraviolet and visible absorption spectra were obtained using a Cary 118 spectrophotometer.

NADH:ubiquinone oxidoreductase activity was measured at 30°C by following ubiquinone dependent NADH oxidation at 340 nm in a 1 mL reaction mixture containing 50 mM Tes buffer, pH 7.5, 250 μM NADH, 40 μM FAD, enzyme and 50 μM ubiquinone-1. The reaction was started by the addition of enzyme and rates were calculated by using $\Delta\varepsilon$ = 6810 M^{-1} cm^{-1} (Schatz and Racker, 1966). NADH oxidase activity was also measured spectrophotometrically by using the system described above, except that ubiquinone was not added and the rates were calculated using $\Delta\varepsilon$ = 6220 M^{-1} cm^{-1}. The oxidase here refers to the activity of the intact respiratory chain in oxidizing NADH and reducing endogenous ubiquinone-8 present in E. coli membranes at 2.2 nmole mg^{-1} protein (Wallace and Young, 1977b). Electrons are transported through the respiratory chain until they reach the KCN inhibitable terminal oxidase (the site of oxygen reduction). The mechanism of the oxidase in the respiratory chain is distinct from the oxidase observed with the purified enzyme. In the case of IY13 membranes the NADH oxidase to NADH:ubiquinone-1 oxidoreductase activity ratio is about 0.4-1.0 whereas with the purified enzyme the ratio is ~0.01. With the purified enzyme the exact mechanism whereby the enzyme and NADH and oxygen interact to oxidize NADH is unknown although FAD involvement is postulated.

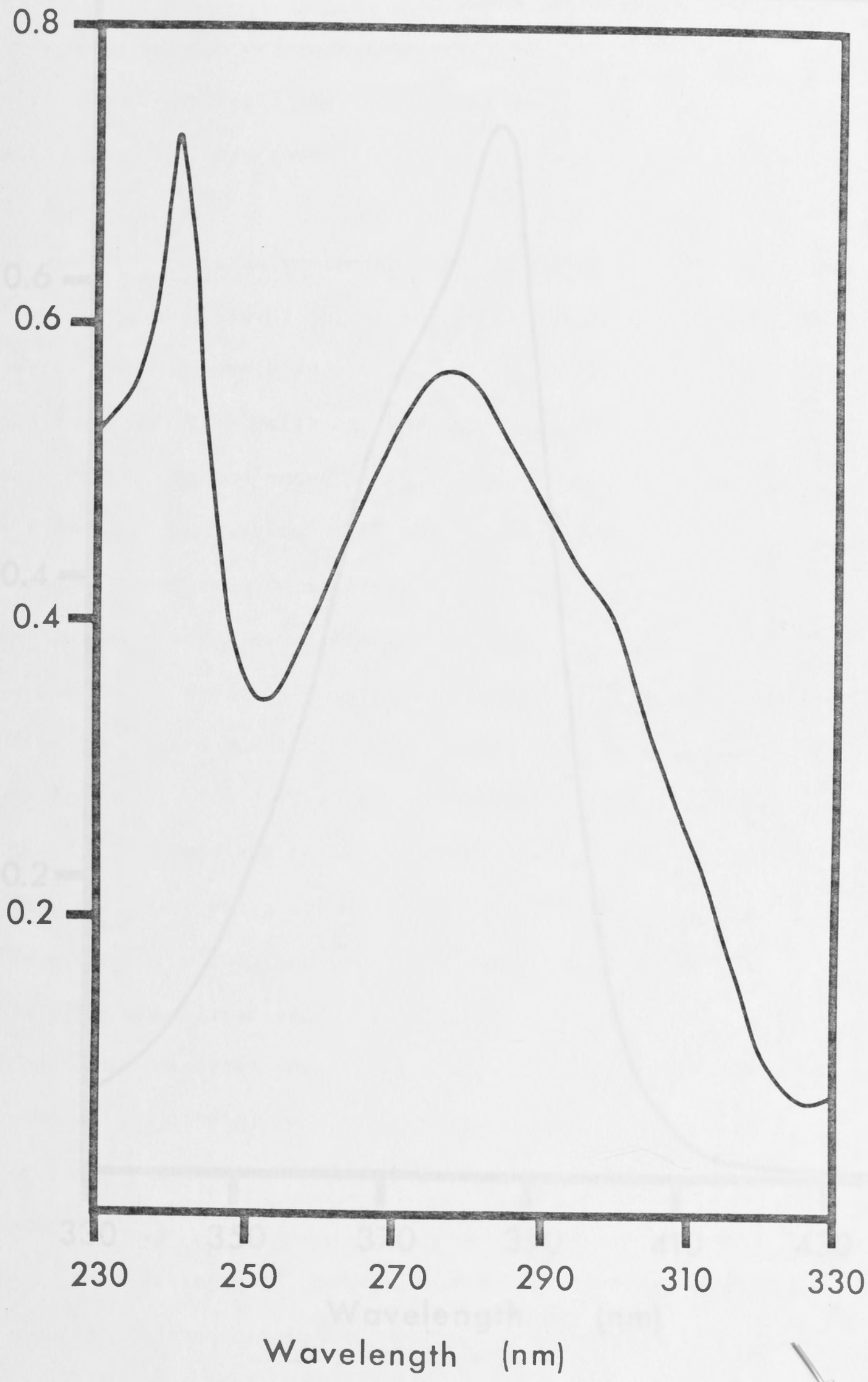
Stock ubiquinone-1 (Q-1), ubiquinone-3 (Q-3) and ubiquinone-8 (Q-8) solutions of 20 mM concentration in redistilled absolute ethanol were prepared and standardized spectrophotometrically as described previously (Crane and Barr, 1971).

NADH:$Fe(CN)_6^{3-}$ oxidoreductase activity was measured at 420 nm and 30°C, using $\Delta\varepsilon$ = 1000 M^{-1} cm^{-1}. The 1 mL reaction mixture contained 50 mM Tes buffer, pH 7.5, 250 µM NADH, 40 µM FAD, enzyme and 1 mM $K_3Fe(CN)_6$. The reaction was started by the addition of the electron acceptor. When membranes were assayed for either NADH:ubiquinone or NADH:$Fe(CN)_6^{3-}$ oxidoreductase activity, 3 mM KCN was incubated for 3 minutes at 30°C with the membranes prior to the addition of electron acceptor. This resulted in >90% inhibition of the NADH oxidase activity.

Because of potential turbidity problems, NADH:ubiquinone-8 oxidoreductase activity assays involving reconstituted vesicles were performed using an Aminco Chance DW2 dual wavelength spectrophotometer. The assays with the Aminco spectrophotometer were monitored in the dual wavelength mode at sample and reference wavelengths of 340 nm and 395 nm respectively and a spectral bandpass of 3 nm. The reference wavelength was chosen because this was the closest wavelength to the sample wavelength that did not undergo a significant change in absorbance upon NADH oxidation. Assays that were done in the presence of DMK-1 (demethylmenaquinone-1) diazide used a reference wavelength of 410 nm to avoid the strong absorption peak at 385 nm (ε = 28400 M^{-1} cm^{-1}, see Figure 3-1 and 3-2 for spectra) with this compound.

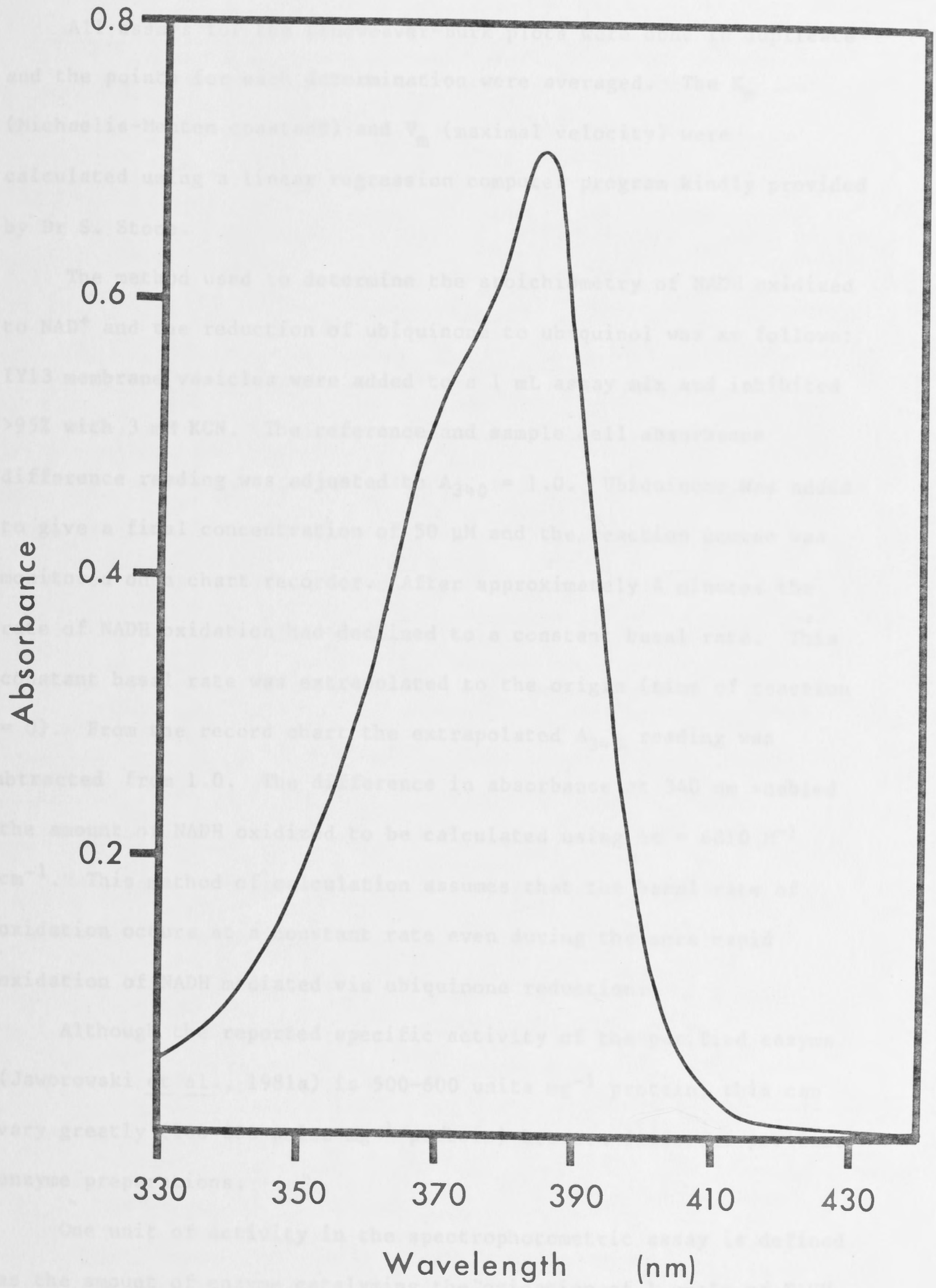
DMK-1 diazide, final concentration 150 µM, was incubated at various times in the assay buffer in the presence of NADH and enzyme and the reaction was initiated by the addition of ubiquinone. In the case of the inhibition results presented in the tables in this chapter, DMK-1 diazide was preincubated with the enzyme for 5 minutes and HQNO, final concentration 12 µM, was preincubated for 3 minutes.

Figure 3-1. Ultraviolet Absorption Spectrum of DMK-1 Diazide



Spectrum of DMK-1 diazide at 50 μM concentration in absolute ethanol.

Figure 3-2. Visible Absorption Spectrum of DMK-1 Diazide



Spectrum of DMK-1 diazide at 50 μM concentration in absolute ethanol.

All assays for the Lineweaver-Burk plots were done in duplicate and the points for each determination were averaged. The K_m (Michaelis-Menten constant) and V_m (maximal velocity) were calculated using a linear regression computer program kindly provided by Dr S. Stone.

The method used to determine the stoichiometry of NADH oxidized to NAD^+ and the reduction of ubiquinone to ubiquinol was as follows: IY13 membrane vesicles were added to a 1 mL assay mix and inhibited >95% with 3 mM KCN. The reference and sample cell absorbance difference reading was adjusted to $A_{340} = 1.0$. Ubiquinone was added to give a final concentration of 50 µM and the reaction course was monitored on a chart recorder. After approximately 4 minutes the rate of NADH oxidation had declined to a constant basal rate. This constant basal rate was extrapolated to the origin (time of reaction = 0). From the record chart the extrapolated A_{340} reading was subtracted from 1.0. The difference in absorbance at 340 nm enabled the amount of NADH oxidized to be calculated using $\Delta\varepsilon = 6810\ M^{-1}\ cm^{-1}$. This method of calculation assumes that the basal rate of oxidation occurs at a constant rate even during the more rapid oxidation of NADH mediated via ubiquinone reduction.

Although the reported specific activity of the purified enzyme (Jaworowski et al., 1981a) is 500-600 units mg^{-1} protein, this can vary greatly (100-600 units mg^{-1} protein) between different purified enzyme preparations.

One unit of activity in the spectrophotometric assay is defined as the amount of enzyme catalyzing the oxidation of 1 µmole of NADH min^{-1}. Specific activity is defined as units per mg of protein.

Protein Estimation

Protein concentrations were determined by the method of Lowry, Rosebrough, Farr and Randall (1951) using defatted bovine serum albumin (BSA) as standard. BSA stock solutions, nominally 10 mg mL^{-1}, were standardized spectrophotometrically using $A^{1\%}_{1cm}$, 280 nm = 6.7.

Synthesis of DMK-1 diazide

DMK-1 diazide was kindly supplied by Dr H.D. Campbell. It was synthesized according to the method of Reid and Dietrich (1961). The DMK-1 diazide was purified by thin layer chromatography before use. Solutions of the purified compound in redistilled ethanol were stored at $-20^{o}C$. No changes in the ultraviolet and visible spectra of the compound were observed after storage over several months (Figures 3-1 and 3-2).

Preparation of Membranes

Membranes were prepared at $4^{o}C$. Cells were harvested by centrifugation and washed once with STM buffer (0.25 M sucrose, 0.1 M Tes, 0.02 M magnesium acetate, pH 7.5). The cells were resuspended in buffer at 1 g wet weight/3 mL STM buffer and disrupted using a Ribi cell fractionator at 20000 $lb/inch^2$. The cell smash was centrifuged at 15000 rpm for 1 hour in a Sorvall SS-34 rotor. The supernatant was centrifuged for 2 hours at 60000 rpm in a Spinco 60Ti rotor. The pellet, containing crude membranes was resuspended to a protein concentration of ~40 mg mL^{-1}. The resuspended membrane vesicles were centrifuged as before and the pelleted membrane vesicles were resuspended to a protein concentration of approximately 35 mg mL^{-1}.

Membrane vesicles were snap frozen using liquid nitrogen and stored at -20°C.

Bacterial Strains

The ndh mutant strain IY12 (thi his ilv trp rpsL ndh), a derivative of E. coli K12, has been described previously (Young et al., 1978). IY13 (thi his ilv trp rpsL) is an isogenic transformant of IY12.

RESULTS AND DISCUSSION

The Stoichiometry of Ubiquinone Reduction

The stoichiometry of NADH oxidized to NAD^+ and the corresponding amount of ubiquinone reduced to ubiquinol was determined with purified enzyme and IY13 membrane vesicles (Table 3-1). It was found to be ~1:1 for ubiquinone-1 with pure enzyme, and for ubiquinone-1 and ubiquinone-3 with IY13 membrane vesicles (terminal oxidase inhibited with KCN). The 1:1 stoichiometry is as expected for the oxidation of NADH to NAD^+ and the concomitant reduction of ubiquinone to ubiquinol. However, it does not preclude the involvement of ubisemiquinone as an intermediate since the disproportionation reaction would be extremely rapid (Krȍger and Klingenberg, 1973a).

Michaelis Constants for Different Ubiquinone Homologues

Little is known about the mechanism of the reaction catalyzed by the respiratory NADH dehydrogenase. The stereospecificity of the reaction has been examined and it has been shown that hydride

Table 3-1. Stoichiometry for the Oxidation of NADH to NAD^+ and the Concomitant Reduction of Ubiquinone to Ubiquinol

Ubiquinone Substrate	nmol NADH oxidized / nmol Q reduced	Preparation
Q-1	1.0	purified enzyme
Q-1	1.1	IY13 membranes
Q-3	1.1	IY13 membranes

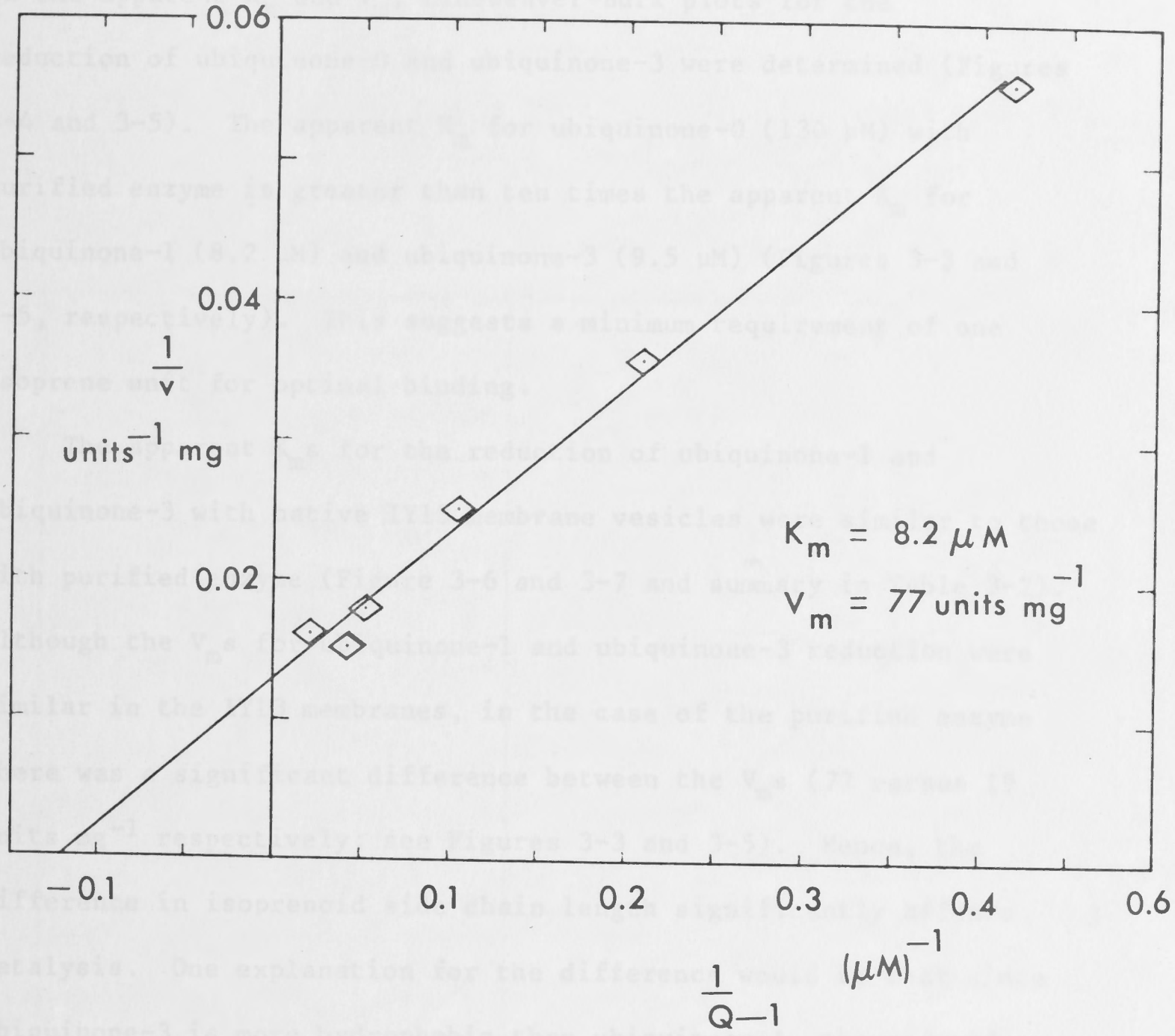
The stoichiometric determinations were performed as described in the Materials and Methods. 47 µg protein of membrane vesicles were added in each assay. Purified enzyme (0.32 µg was assayed in the same manner except that the addition of KCN was omitted.

transfer occurs from the 4B position of the nicotinamide ring of NADH (Campbell, unpublished). At the present time no further information is available concerning the mechanism of FAD reduction or the mechanism of electron transfer to ubiquinone.

If the respiratory NADH dehydrogenase possesses a specific ubiquinone binding site then the reduction of ubiquinone should follow a hyperbolic kinetic course. However, if ubiquinone interacts in a non-specific manner, ie random collision, then ubiquinone reduction should follow a linear kinetic course. It was shown that the purified enzyme has a linear Lineweaver-Burk plot for the reduction of ubiquinone-1 (Figure 3-3). The apparent Michaelis constant for NADH oxidation by purified NADH:ubiquinone oxidoreductase under the assay conditions used was previously estimated to be ~57 μM from Lineweaver-Burk plots over the range 7-150 μmole NADH (Jaworowski et al., 1981a). The NADH oxidase activity of IY13 membrane vesicles exhibited an apparent K_m for NADH of ~55 μM, similar to the value obtained for the purified enzyme (Jaworowski et al., 1981a). In the present experiments NADH was used at 250 μM concentration to ensure saturation.

It is clear from Figure 3-3, that the purified enzyme has a specific ubiquinone binding site and high catalytic activity. The reported specific activity of the E. coli NADH:ubiquinone oxidoreductase is considerably greater than that of complex I, 500-600 versus 16 μmole NADH oxidized min^{-1} mg^{-1} protein (Jaworowski et al., 1981a; Ragan, 1976a). However, the specific activity of different purified E. coli enzyme preparations can vary greatly (100-600 units mg^{-1} min^{-1}). The apparent K_m for ubiquinone-1 reduction by E. coli purified enzyme (8.2 μM) is lower than that recorded for complex I (44 μM; Ragan, 1976a).

Figure 3-3. Lineweaver-Burk Plot for the Reduction of Ubiquinone-1 by Purified NADH:ubiquinone Oxidoreductase



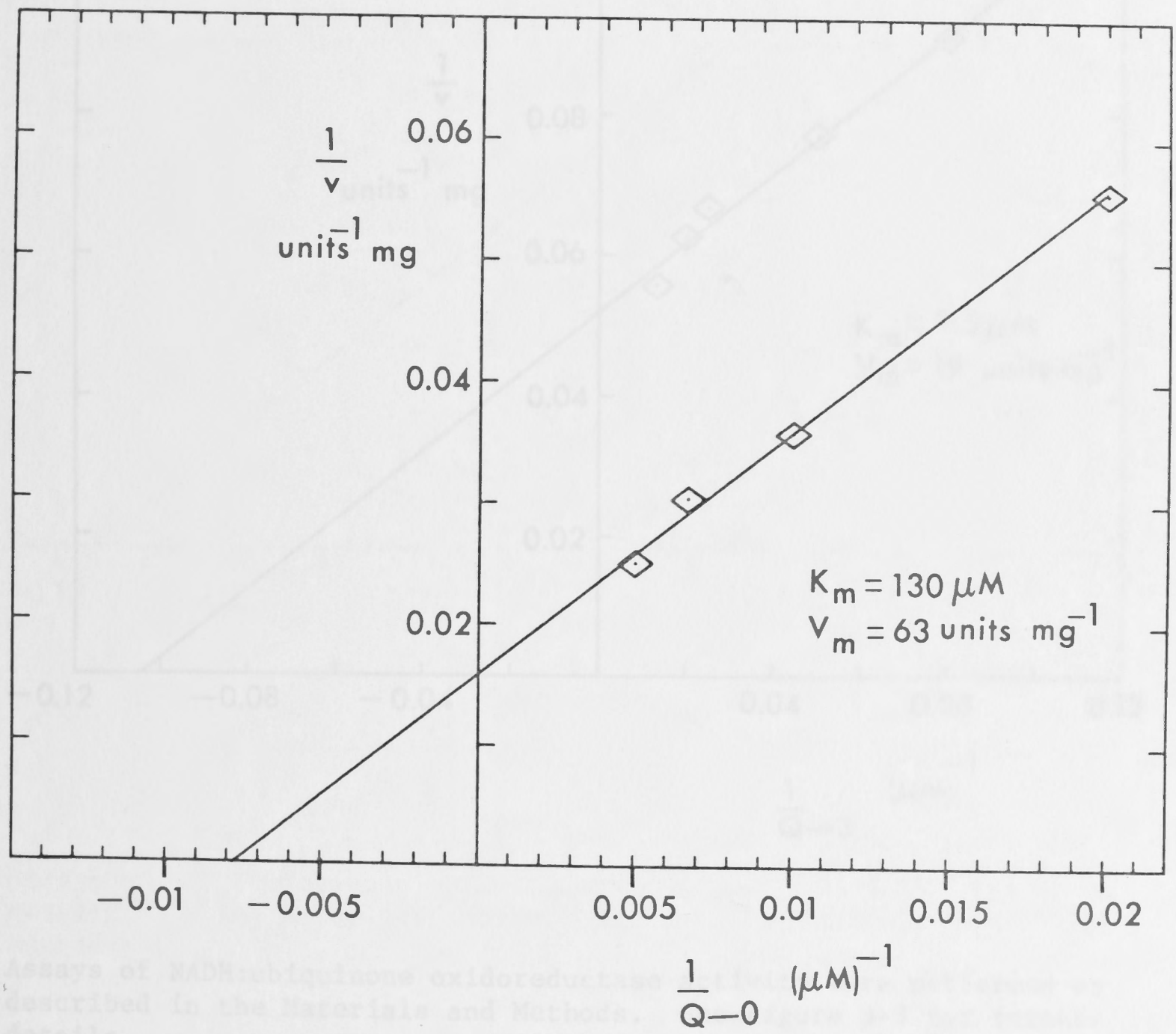
Assays of NADH:ubiquinone oxidoreductase activity were performed as described in the Materials and Methods. 0.32 µg of purified enzyme was added to each assay.

In order to examine the effect of ubiquinone side chain length on the apparent K_m and V_m, Lineweaver-Burk plots for the reduction of ubiquinone-0 and ubiquinone-3 were determined (Figures 3-4 and 3-5). The apparent K_m for ubiquinone-0 (130 μM) with purified enzyme is greater than ten times the apparent K_m for ubiquinone-1 (8.2 μM) and ubiquinone-3 (9.5 μM) (Figures 3-3 and 3-5, respectively). This suggests a minimum requirement of one isoprene unit for optimal binding.

The apparent K_ms for the reduction of ubiquinone-1 and ubiquinone-3 with native IY13 membrane vesicles were similar to those with purified enzyme (Figure 3-6 and 3-7 and summary in Table 3-2). Although the V_ms for ubiquinone-1 and ubiquinone-3 reduction were similar in the IY13 membranes, in the case of the purified enzyme there was a significant difference between the V_ms (77 versus 19 units mg^{-1} respectively; see Figures 3-3 and 3-5). Hence, the difference in isoprenoid side chain length significantly affects catalysis. One explanation for the difference would be that since ubiquinone-3 is more hydrophobic than ubiquinone-1, the reduced product (ubiquinol-3) may not partition as readily back into the aqueous phase but would remain in a position on the enzyme which slows down the free passage of unreacted substrate to the active site. Attempts were also made to determine these parameters for the naturally occurring ubiquinone homologue (ubiquinone-8) but too great a variability was found presumably due to the insolubility of this compound.

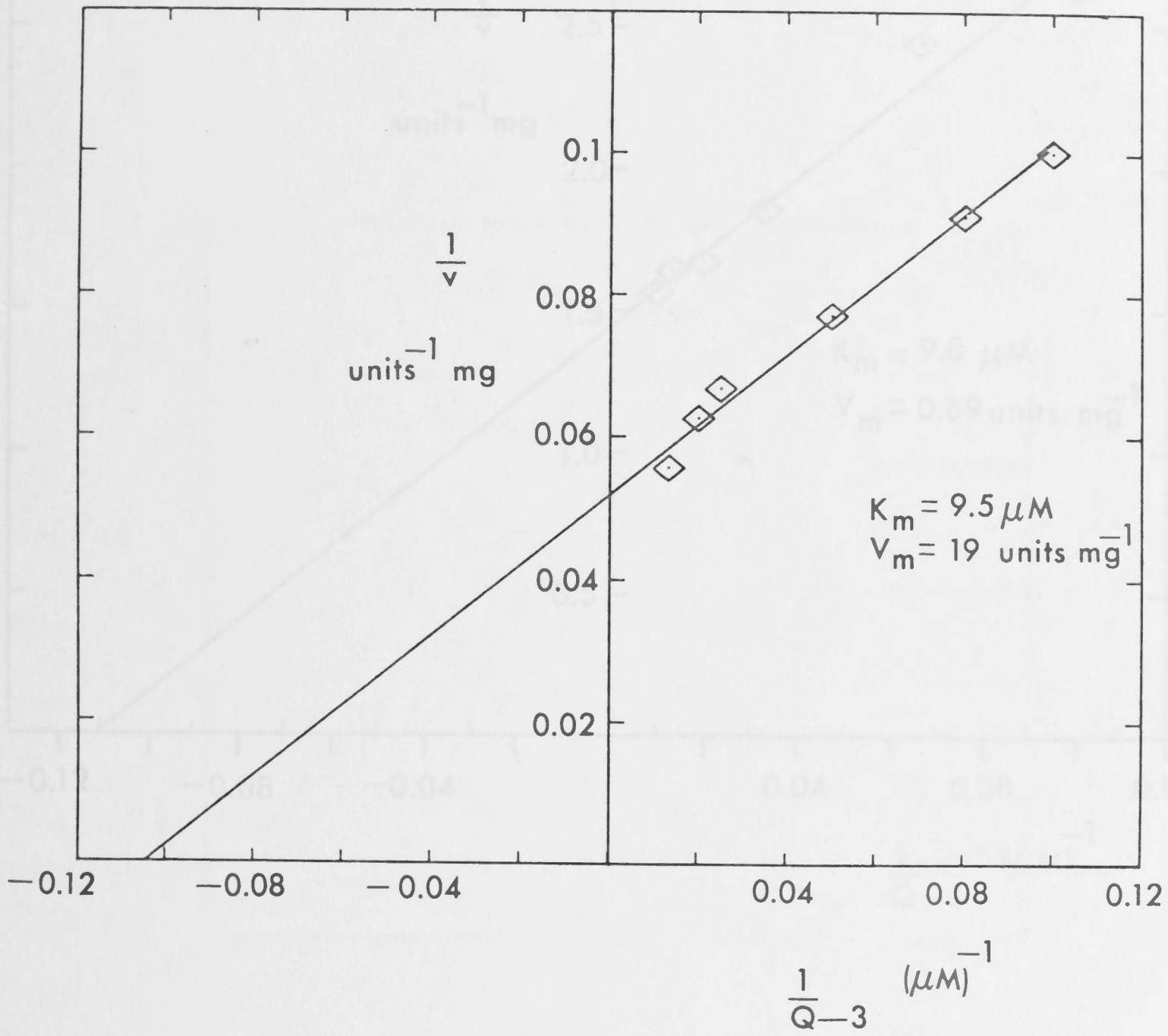
It is obvious that the use of hydrophobic substrates (eg ubiquinone long sidechain homologues) presents difficulties for assays. One possible means of increasing ubiquinone reductase

Figure 3-4. Lineweaver-Burk Plot for the Reduction of Ubiquinone-0 by Purified NADH:ubiquinone Oxidoreductase



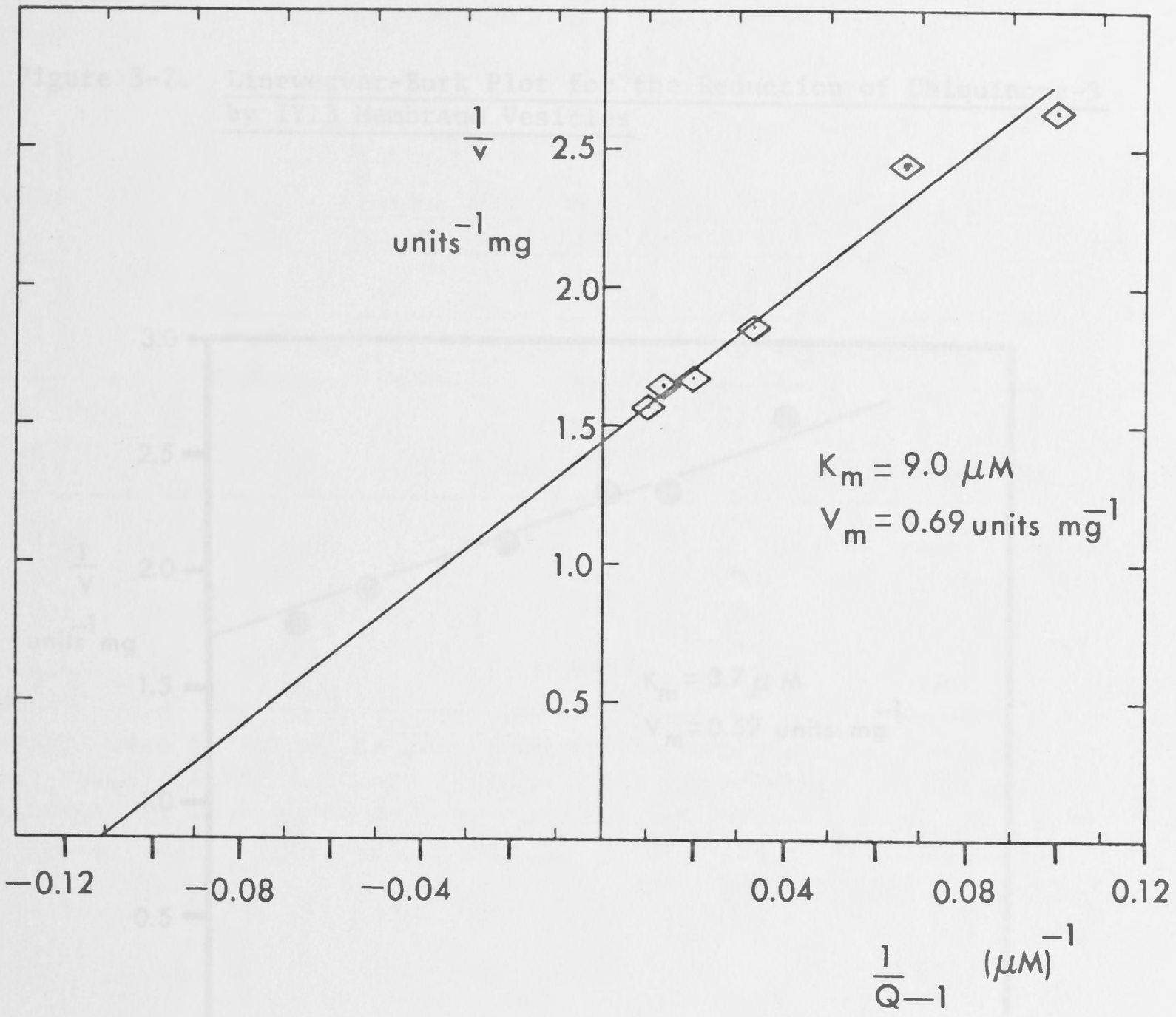
Assays of NADH:ubiquinone oxidoreductase activity were performed as described in the Materials and Methods. See Figure 3-3 for further details.

Figure 3-5. Lineweaver-Burk Plot for the Reduction of Ubiquinone-3 by Purified NADH:ubiquinone Oxidoreductase



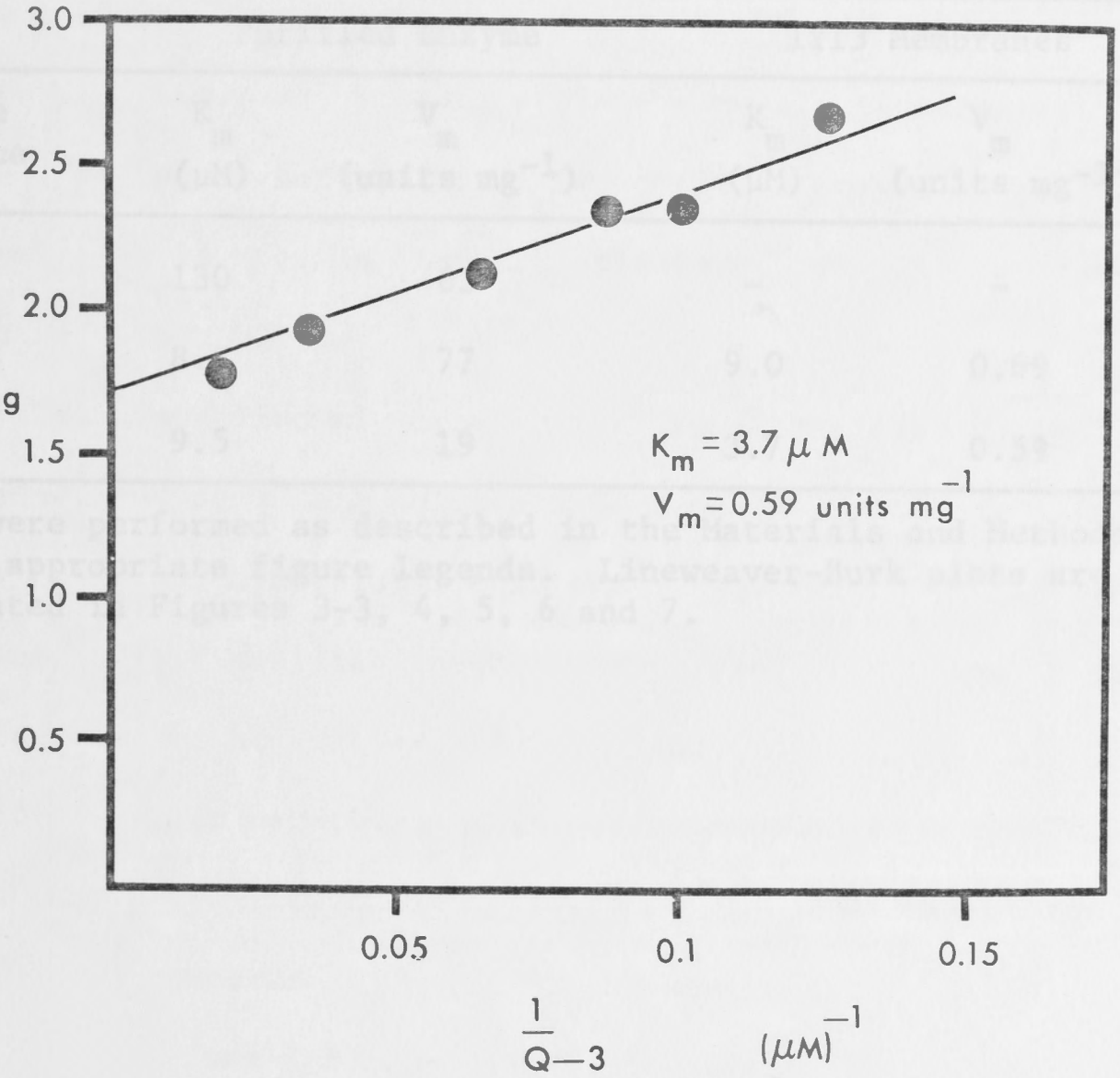
Assays of NADH:ubiquinone oxidoreductase activity were performed as described in the Materials and Methods. See Figure 3-3 for further details.

Figure 3-6. Lineweaver-Burk Plot for the Reduction of Ubiquinone-1 by IY13 Membrane Vesicles



Assays of NADH:ubiquinone oxidoreductase activity were performed as described in the Materials and Methods. 47 μg protein was added to each assay.

Figure 3-7. Lineweaver-Burk Plot for the Reduction of Ubiquinone-3 by IY13 Membrane Vesicles



Assays of NADH:ubiquinone oxidoreductase activity were performed as described in the text. See Figure 3-6 for further details.

Table 3-2. K_m and V_m Values Determined Using Various Ubiquinones as Substrates with Purified Enzyme and IY13 Membranes

	Purified Enzyme		IY13 Membranes	
Quinone Homologue	K_m (μM)	V_m (units mg^{-1})	K_m (μM)	V_m (units mg^{-1})
Q-0	130	63	-	-
Q-1	8.2	77	9.0	0.69
Q-3	9.5	19	3.7	0.59

Assays were performed as described in the Materials and Methods and the appropriate figure legends. Lineweaver-Burk plots are illustrated in Figures 3-3, 4, 5, 6 and 7.

activity is to perform assays with detergent present which would increase the solubility of hydrophobic substrates.

The effect of cholate on the ubiquinone-1 and ubiquinone-3 oxidoreductase activities of purified enzyme and the NADH oxidase activity of IY13 membrane vesicles was assessed. It was clear that the presence of 0.5-1.0% (w/v) cholate leads to stimulation of the ubiquinone-3 reductase activity especially in the case of the purified enzyme. However, at this cholate concentration range, the ubiquinone-1 reductase activities of the purified enzyme and IY13 membrane vesicles as well as the NADH oxidase of the IY13 membranes were inhibited (~60-80%).

A more membrane-like microenvironment could facilitate release of the product and stimulate ubiquinone-3 reductase activity. In an attempt to test this hypothesis, pure enzyme was reconstituted into soybean phospholipid vesicles (neutral lipid free) according to the cholate dialysis method (Racker, 1979; Hokin, 1981; Eytan, 1982; see Chapter 4 for further details). When the reconstituted enzyme was assayed for ubiquinone reductase activity it was demonstrated that the ubiquinone-3 reductase was ~70% of the observed ubiquinone-1 reductase activity (see Table 3-5). This relationship is very similar to the relative V_ms determined for ubiquinone-1 and ubiquinone-3 reduction with IY13 membranes (Table 3-2) and contrasts with the relatively low ubiquinone-3 reductase activity of the pure enzyme (Table 3-2).

Action of Inhibitors

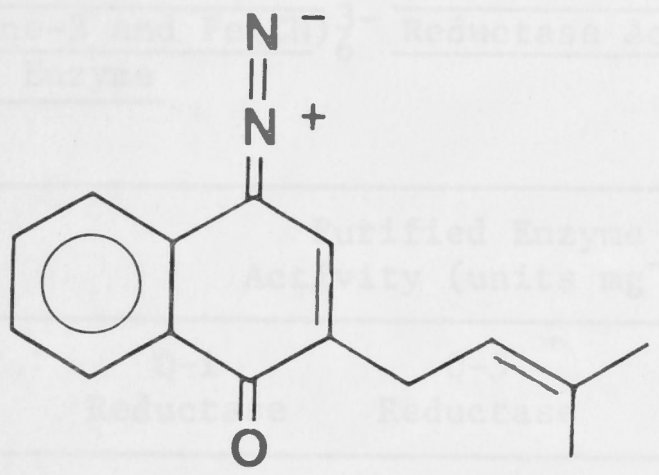
It was of interest to examine the effect on the ubiquinone reductase activities of purified enzyme and IY13 membrane vesicles of

inhibitors known to inhibit electron transport. Although a number of inhibitors were considered, including piericidin and rotenone, preliminary experiments indicated that HQNO and DMK-1 diazide were the most promising. Both HQNO and DMK-1 diazide have structures which resemble the naphthoquinones (Figures 3-8). Although HQNO has been used as a potent inhibitor of mitochondrial NADH and succinate oxidase activities (Nijs, 1967; Brandon, Brocklehurst and Lee, 1972; Ark and Berden, 1977; Burger, 1980; Zhu et al., 1982) it has not been explicitly claimed that HQNO is a ubiquinone analogue or that it specifically acts at a ubiquinone binding site. Nonetheless, Cox et al. (1970) also reported that the NADH oxidase of E. coli was inhibited at low concentrations (16 μM) of HQNO and that inhibition was reversed by the addition of ubiquinone-1.

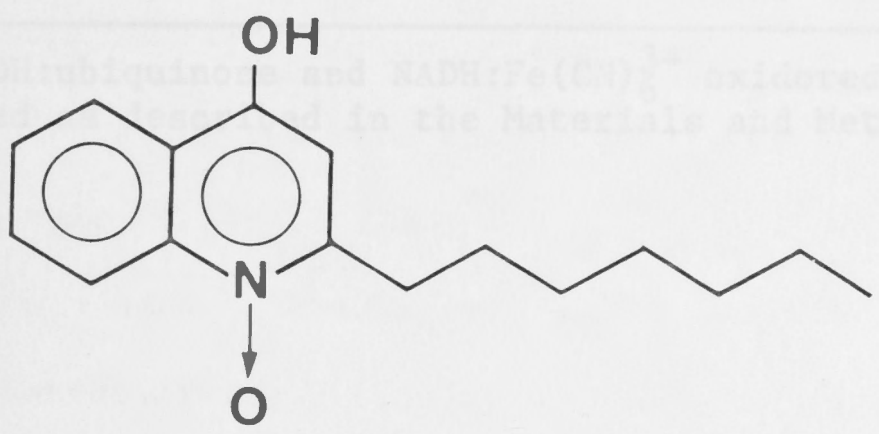
DMK-1 diazide was designed and synthesized by Dr H.D. Campbell. The author has undertaken its preliminary characterization as an inhibitor of electron transfer in the E. coli respiratory chain.

The effect of HQNO and DMK-1 diazide on the ubiquinone-1, ubiquinone-3 and $Fe(CN)_6^{3-}$ reductase activities of the purified enzyme is shown in Table 3-3. Both inhibitors gave strong inhibition of ubiquinone-1 reductase activity. HQNO was ineffective as an inhibitor of the NADH:$Fe(CN)_6^{3-}$ oxidoreductase activity of the purified enzyme, whereas DMK-1 diazide reduced this activity to less than 5% of the untreated enzyme. This result infers that the site of action of the two inhibitors is not the same. The failure of HQNO to inhibit the NADH:$Fe(CN)_6^{3-}$ oxidoreductase activity of the E. coli enzyme while being a potent inhibitor of NADH:ubiquinone-1 oxidoreductase activity is analogous to the action of rotenone in mammalian mitochondria (Hatefi and Stiggall, 1976).

Figure 3-8. Structures of HQNO and DMK-1 Diazide



DMK-1 diazide
(demethylmenaquinone-1 diazide)



HQNO
(2-n-heptyl-4-hydroxyquinoline-N-oxide)

Table 3-3. HQNO and DMK-1 Diazide as Inhibitors of Ubiquinone-1, Ubiquinone-3 and $Fe(CN)_6^{3-}$ Reductase Activities of Purified Enzyme

Inhibitor	Purified Enzyme Activity (units mg^{-1})		
	Q-1 Reductase	Q-3 Reductase	$Fe(CN)_6^{3-}$ Reductase
-	270	27	89
HQNO	25	23	91
DMK-1 diazide	12.8	-	2.6

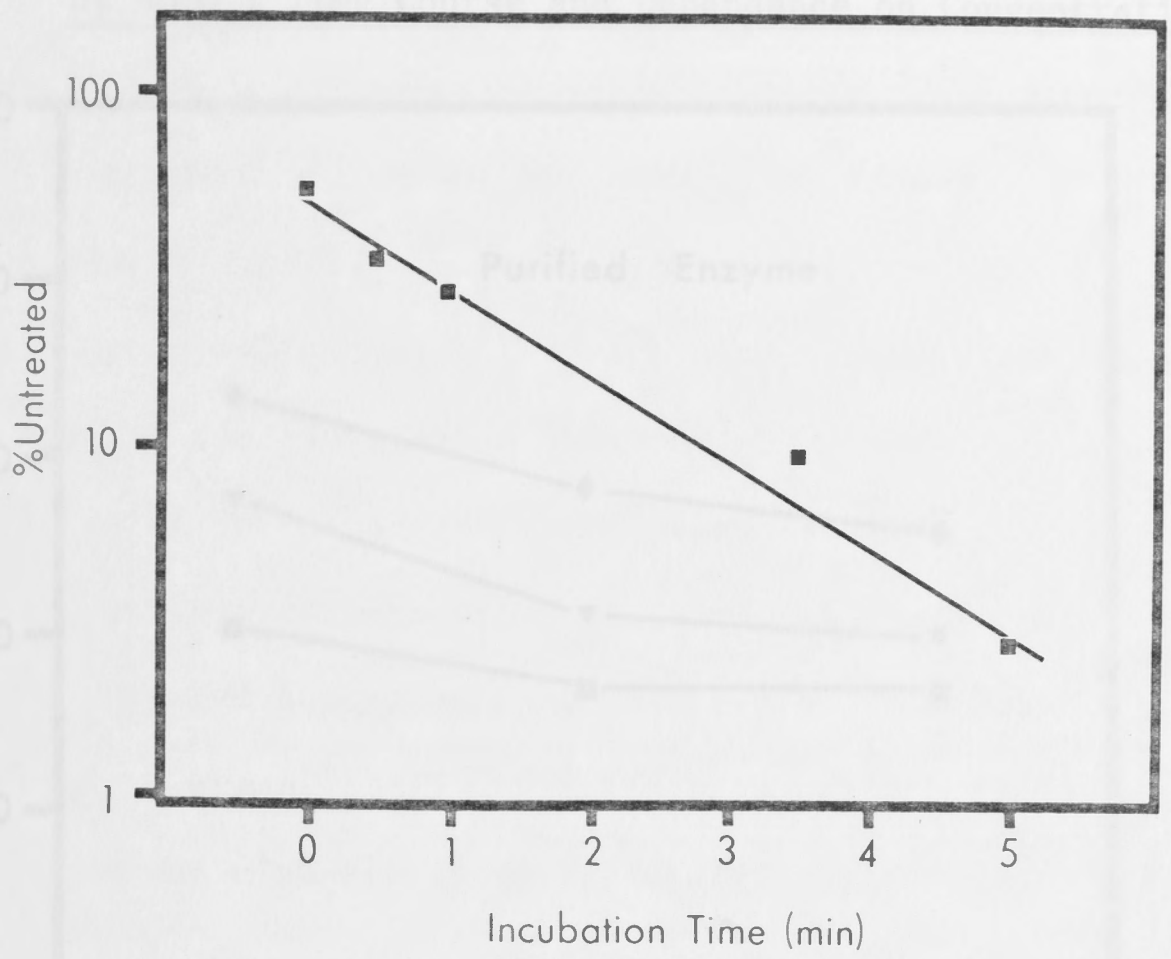
Assays of NADH:ubiquinone and NADH:$Fe(CN)_6^{3-}$ oxidoreductase were performed as described in the Materials and Methods.

A time course of the inhibition of NADH:ubiquinone-1 and NADH:$Fe(CN)_6^{3-}$ oxidoreductase activities of the purified enzyme by DMK-1 diazide is illustrated in Figure 3-9. The slope of the lines in Figure 3-9 differ by <10% which suggests that DMK-1 diazide affects both the NADH:ubiquinone-1 and NADH:$Fe(CN)_6^{3-}$ oxidoreductase activities in the same manner. The time courses and the dependence on concentration of HQNO and DMK-1 diazide inhibition of NADH:ubiquinone-1 oxidoreductase activity were significantly different (Figures 3-9 and 3-10). Concentrations of HQNO greater than 12 μM, were incompatible with the assay conditions. However, as shown in Figure 3-10, at an HQNO concentration of 12 μM and after 2-4 minutes incubation with pure enzyme or IY13 membrane vesicles, the extent of inhibition (~70% and 75%, respectively) reached a plateau. The residual 'HQNO insensitive' (~20-30%) activity is reminiscent of the mitochondrial rotenone insensitive portion of the NADH:ubiquinone oxidoreductase (see Introduction). No such effect was observed with DMK-1 diazide as an inhibitor of the NADH:ubiquinone-1 oxidoreductase activity of purified enzyme (Figure 3-9).

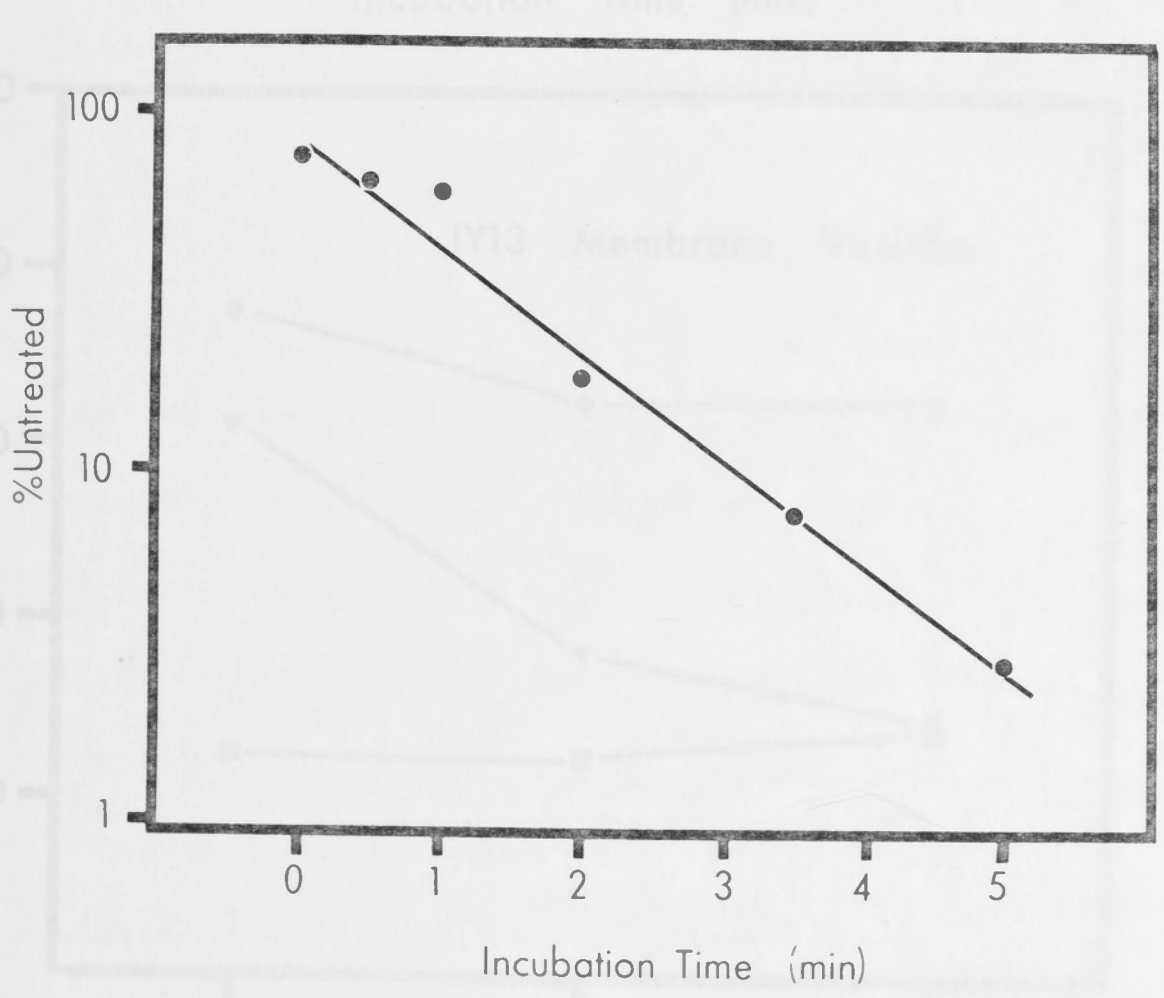
When ubiquinone-3 was used as a substrate of the purified enzyme it was found that the extent of inhibition by HQNO was markedly reduced (Table 3-3). This effect was not observed with reconstituted enzyme or normal membranes where the rates with ubiquinone-3 as substrate more closely approximated those with ubiquinone-1 (Tables 3-4 and 3-5).

In agreement with previous reports (Jones, 1967; Bragg and Hou, 1967a; Cox et al., 1970) HQNO gave strong inhibition of the NADH oxidase activity of IY13 membrane vesicles at similar concentrations to those used with the purified enzyme (Table 3-4). In this case it

Figure 3-9. Inhibition of NADH:$Fe(CN)_6^{3-}$ Oxidoreductase Activity of Purified Enzyme by DMK-1 Diazide

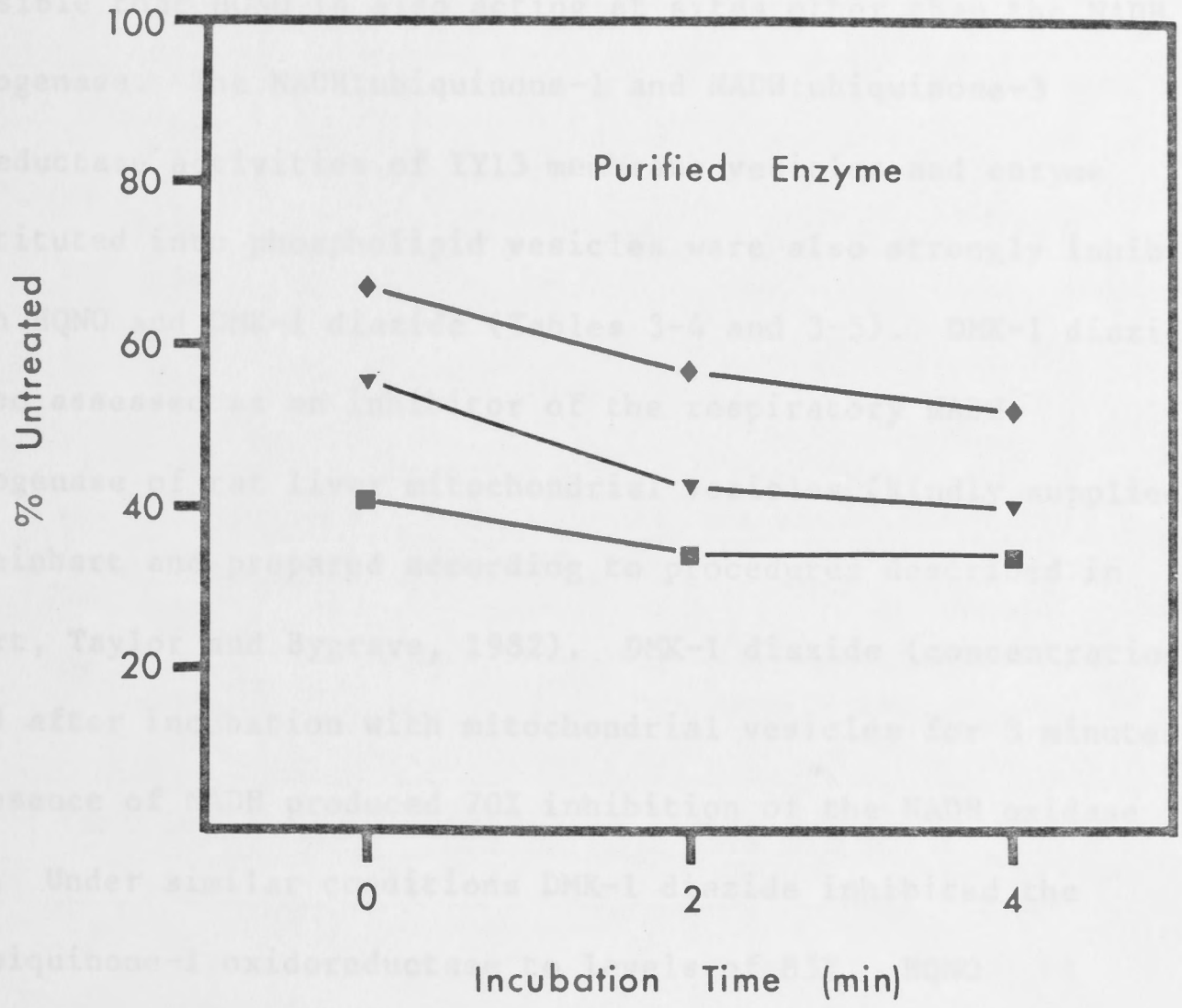


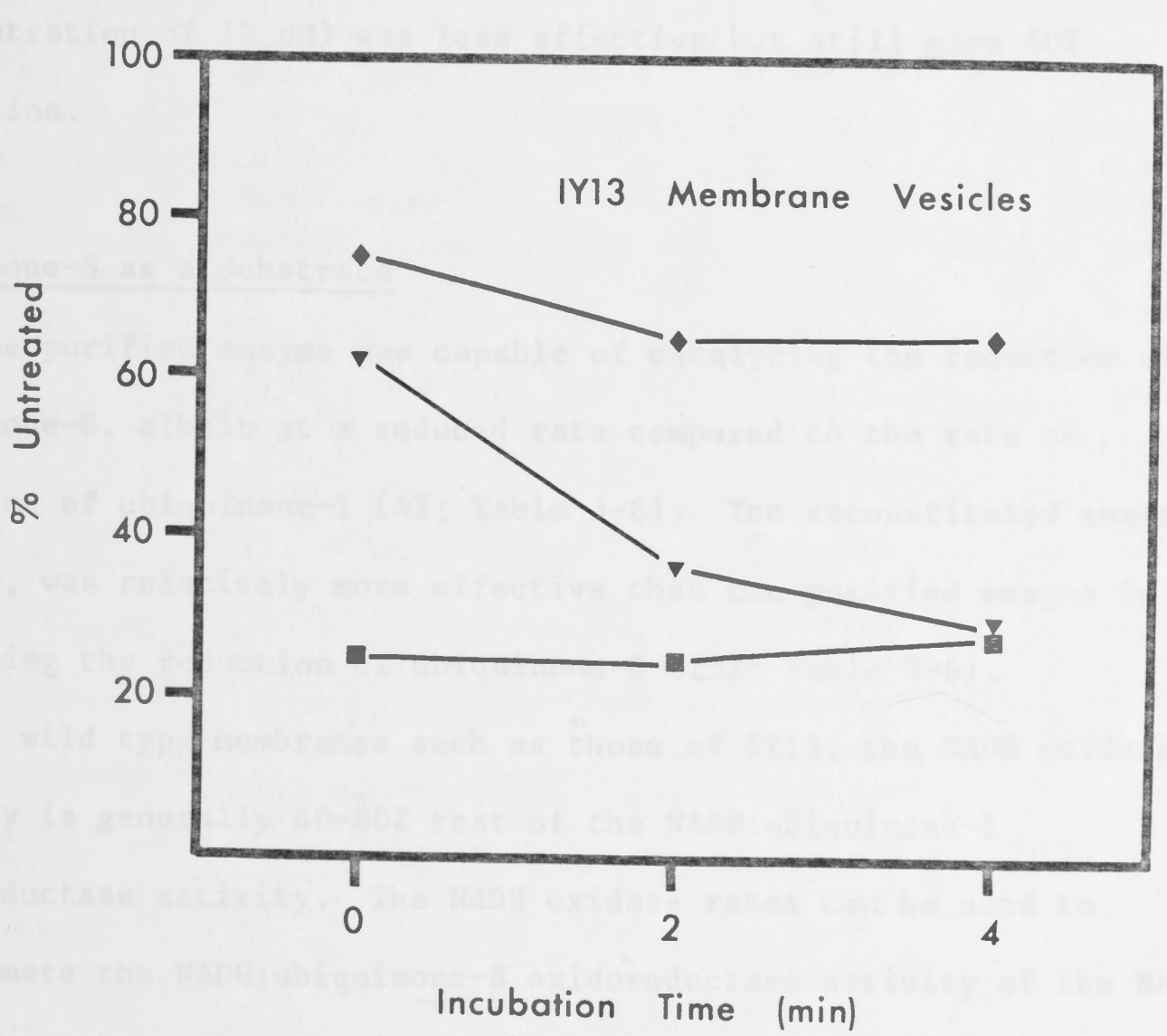
Inhibition of NADH:ubiquinone-1 Oxidoreductase Activity of Purified Enzyme by DMK-1 Diazide



The assays were performed as described in the Materials and Methods. The only alteration to the procedure was that the time of incubation in the presence of DMK-1 diazide was varied.

Figure 3-10. Inhibition of the NADH:ubiquinone-1 Oxidoreductase Activity of Purified Enzyme and IY13 Membrane Vesicles by HQNO : Time Course and Dependence on Concentration





The assays were performed as described in the Materials and Methods. The different symbols represent various concentrations of HQNO: (◆) 1.2 μM, (▼) 6 μM, and (■) 12 μM.

is possible that HQNO is also acting at sites other than the NADH dehydrogenase. The NADH:ubiquinone-1 and NADH:ubiquinone-3 oxidoreductase activities of IY13 membrane vesicles and enzyme reconstituted into phospholipid vesicles were also strongly inhibited by both HQNO and DMK-1 diazide (Tables 3-4 and 3-5). DMK-1 diazide was also assessed as an inhibitor of the respiratory NADH dehydrogenase of rat liver mitochondrial vesicles (kindly supplied by P.H. Reinhart and prepared according to procedures described in Reinhart, Taylor and Bygrave, 1982). DMK-1 diazide (concentration of 150 μM) after incubation with mitochondrial vesicles for 3 minutes in the presence of NADH produced 70% inhibition of the NADH oxidase levels. Under similar conditions DMK-1 diazide inhibited the NADH:ubiquinone-1 oxidoreductase to levels of 85%. HQNO (concentration of 12 μM) was less effective but still gave 50% inhibition.

Ubiquinone-8 as a Substrate

The purified enzyme was capable of catalyzing the reduction of ubiquinone-8, albeit at a reduced rate compared to the rate of reduction of ubiquinone-1 (4%; Table 3-6). The reconstituted enzyme, however, was relatively more effective than the purified enzyme in catalyzing the reduction of ubiquinone-8 (25%; Table 3-6).

In wild type membranes such as those of IY13, the NADH oxidase activity is generally 40-80% that of the NADH:ubiquinone-1 oxidoreductase activity. The NADH oxidase rates can be used to approximate the NADH:ubiquinone-8 oxidoreductase activity of the NADH dehydrogenase *in vivo* since amplification experiments indicate that the dehydrogenase is rate limiting in natural membranes. Thus, the

Table 3-4. Inhibition of NADH Oxidase, NADH:ubiquinone-1 and NADH: ubiquinone-3 Oxidoreductase Activities of IY13 Membrane Vesicles by HQNO and DMK-1 diazide

	IY13 Membrane Vesicles Activity (units mg^{-1})		
Inhibitor	Oxidase	Q-1 Reductase	Q-3 Reductase
-	0.27	0.72	0.51
HQNO	0.01	0.15	0.18
DMK-1 diazide	0.09	0.06	0.11

Assays were performed as described in the Materials and Methods.

Table 3-5. HQNO and DMK-1 Diazide as Inhibitors of NADH:ubiquinone-1 and NADH:ubiquinone-3 Oxidoreductase Activities of Reconstituted Enzyme

	Reconstituted Enzyme Activity (units mL^{-1})	
Inhibitor	Q-1 Reductase	Q-3 Reductase
-	12	7.9
HQNO	3.7	4.4
DMK-1 diazide	1.5	0.9

Assays were performed as described in the Materials and Methods with an Aminco Chance dual wavelength spectrophotometer.

Table 3-6. Effect of Ubiquinone-8 on NADH:ubiquinone-1 Oxidoreductase Activity

Enzyme Preparation	Ubiquinone Reductase Activity[a] Q-1	Q-8	Q-8+Q-1[b]
Purified Enzyme	120	4.9	25
Reconstituted Enzyme[c]	11	2.7	12

[a] Assays were performed as described in the Materials and Methods using Q-1 or Q-8 as acceptor (50 μM concentration). The activity figures are given in units mg^{-1} protein for purified enzyme and units mL^{-1} for reconstituted enzyme.

[b] After the reaction was initiated by the addition of Q-8 (50 μM final), Q-1 (50 μM final) was added and the ubiquinone dependent NADH oxidation was recorded.

[c] Purified enzyme was reconstituted into phospholipid vesicles by the cholate dialysis method (see Materials and Methods Chapter 4).

NADH:ubiquinone-8 oxidoreductase activity measured for purified NADH dehydrogenase reconstituted into phospholipid vesicles is approaching its activity *in vivo*. The hypothesis put forward previously was that the longer chain isoprenoid ubiquinones were able to act specifically with the purified enzyme but solubility in the aqueous phase presented difficulties for release of the reduced ubiquinone. If this explanation is correct then ubiquinone-8 should act as an inhibitor of NADH:ubiquinone-1 oxidoreductase of purified enzyme but act as a substrate in the case of the reconstituted enzyme. This assumes that the ubiquinone homologues are acting at the same active site. The results obtained for the use of ubiquinone-8 as an inhibitor of ubiquinone-1 reductase in various enzyme preparations are shown in Table 3-6. The results were consistent with the hypothesis formulated above and furthermore, with the reconstituted enzyme preparation the presence of ubiquinone-8 prior to the addition of ubiquinone-1 did not lead to inhibition of the ubiquinone-1 reductase (Table 3-6).

SUMMARY

The stoichiometry of NADH oxidized to NAD^+ and the corresponding amount of ubiquinone reduced to ubiquinol was found to be 1:1 with purified enzyme and IY13 membrane vesicles.

The evidence presented in the present work verifies that the purified respiratory NADH dehydrogenase of *E. coli* is capable of reducing ubiquinone-1 at high rates without the involvement of any auxilliary protein(s). The purified enzyme catalyzes the reduction

of various ubiquinone homologues. Apparent K_m values for ubiquinone-1 and ubiquinone-3 are <10 μM with the enzyme as purified and in native E. coli membrane vesicles. After reconstitution into phospholipid vesicles, the enzyme possesses high ubiquinone-8 reductase activity.

Results of kinetic and inhibition studies are consistent with the view that ubiquinone-1, ubiquinone-3 and ubiquinone-8 (the naturally occurring homologue in E. coli membranes) all act at the same catalytic site on the enzyme.

HQNO, a known inhibitor of mitochondrial NADH and succinate oxidase, has been shown to inhibit the NADH oxidase, NADH:ubiquinone-1 and NADH:ubiquinone-3 oxidoreductase activities of IY13 membrane vesicles. HQNO has also been shown to inhibit the NADH:ubiquinone-1 oxidoreductase of purified enzyme as well as the NADH:ubiquinone-1 and NADH:ubiquinone-3 oxidoreductase activities of reconstituted enzyme. It is interesting to note that HQNO was ineffective as an inhibitor of the NADH:$Fe(CN)_6^{3-}$ oxidoreductase activity of purified enzyme.

A novel inhibitor of ubiquinone reductase activity, DMK-1 diazide, was synthesized (by Dr H.D. Campbell) and its effect on various enzymic activities of purified enzyme and IY13 membrane vesicles was assessed. DMK-1 diazide was shown to inhibit both the E. coli NADH oxidase, NADH:ubiquinone-1 oxidoreductase and NADH:$Fe(CN)_6^{3-}$ oxidoreductase activities as well as the rat liver mitochondrial NADH oxidase and NADH:ubiquinone-1 oxidoreductase activities.

These results are consistent with ubiquinone-8 being the immediate electron acceptor for the E. coli respiratory NADH dehydrogenase in vivo.

Chapter 4

Reconstitution of the Respiratory NADH:ubiquinone Oxidoreductase into Natural and Artificial Membranes

INTRODUCTION

The Assembly of Proteins into Biological Membranes

A major stimulus to membrane biology came with the development and exposition of the signal hypothesis to explain the mechanism of protein transfer across membranes (Blobel and Dobberstein, 1975). This hypothesis was further extended to include membrane proteins in general (Rothman and Lenard, 1977). The signal hypothesis seeks to explain the widespread occurrence of hydrophobic N-terminal leader peptides in membrane proteins and secreted proteins. These peptides are 15-30 residues long and are removed rapidly during, or shortly after, the peptides' passage across the membrane. The signal hypothesis suggests that this N-terminal region binds to a receptor in the bilayer and is recognized by a membrane-bound peptide transport system. The ribosome catalyzes the peptide's synthesis and passage through this specific pore. On the opposite face of the bilayer, the emerging leader peptide is removed by a specific protease.

A large body of evidence has been accumulated in support of a number of the postulates outlined in the signal hypothesis of Blobel and Dobberstein (1975). This evidence includes: (a) the isolation of a large number of precursors of secreted proteins from eukaryotes (Kreil, 1981) and prokaryotes (Mutoh, Inokuchi and Mizushima, 1982) which possess an N-terminal signal peptide which is proteolytically cleaved upon excretion, (b) the detection of leader peptidases in eukaryotes (Jackson and Blobel, 1977; Strauss, Zimmerman, Boime, Ashe, Mumford and Alberts, 1979) and prokaryotes (Chang, Blobel and Model, 1978; Mandel and Wickner, 1979; Date and Wickner, 1981), (c)

the detection in E. coli of specific sites in the inner membrane where secreted proteins share a common site of localization and processing (Ito, Bassford and Beckwith, 1981) and (d) the detection of a Signal Recognition Protein in microsomal membranes of dog pancreas (Walter, Ibrahimi and Blobel, 1981) and a 'Docking Protein' (Meyer, 1982; Meyer and Dobberstein, 1980). The latter two proteins are cytoplasmic and are involved in the mechanism of vectorial transfer of secreted proteins across the endoplasmic reticulum membrane (Meyer, 1982).

Although the signal hypothesis has proven to be very valuable, it fails to address a number of important questions. Firstly, the hypothesis stresses the role of translation as a mechanism whereby the nascent peptide passes through the membrane (Blobel and Dobberstein, 1975). However, as pointed out by Schatz (1979), several proteins destined to function in mitochondria are imported from the cytoplasm across the mitochondrial membrane independent of translation. Secondly, the signal hypothesis is ostensibly a proposed mechanism to explain how proteins are secreted through membranes and hence does not adequately address the fact that a large number of membrane proteins are neither secreted nor proteolytically cleaved. How do such proteins enter their respective membranes? Thirdly, ovalbumin has been shown to be a secreted protein yet it does not possess a hydrophobic N-terminal leader sequence (Palmiter, Gagnon and Walsh, 1978).

Wickner (1979) proposed an alternative scheme termed the membrane trigger hypothesis which placed less emphasis upon catalysis in membrane assembly. The central distinctions between this model and the signal hypothesis are the absence of a peptide

transport system and the proposed role of the N-terminal leader peptide. According to the membrane trigger hypothesis, the N-terminal leader peptide plays a part in directing protein folding prior to, or during, interaction of the protein with the lipid bilayer (Wickner, 1979). It is proposed that such interaction triggers spontaneous folding and conformational change of the protein leading to its insertion into the membrane without additional catalysis (ibid.). Subsequently, the N-terminal leader peptide is in many cases removed proteolytically (ibid.). However, a number of E. coli inner membrane proteins have now been sequenced which do not possess any processed N-terminal leader sequence (Table 4-1). Although some of the membrane proteins listed in Table 4-1 have hydrophobic amino acid stretches at the N-terminus (ndh, frdA and lacY), several do not (hisP, malK and frdB).

Engelman and Steitz (1981) proposed an alternative scheme called the helical hairpin hypothesis which differed considerably from the signal and membrane trigger hypotheses. They proposed that the initial event in the secretion of proteins across membranes and their insertion into membranes is the spontaneous penetration of the hydrophobic portion of the bilayer by a helical hairpin. Furthermore, they suggest that secretion and insertion of membrane proteins are spontaneous processes that do not require the participation of additional specific membrane receptors or transport proteins (Engelman and Steitz, 1981; Steitz, Goldman and Engelman, 1982). The major strength of the helical hairpin hypothesis over its predecessors is that it attempts to explain how proteins become membrane bound or secreted through membranes even though they lack a hydrophobic N-terminus or are not proteolytically processed.

Table 4-1. Sequenced *E. coli* Inner-Membrane Proteins which do not possess Processed N-Terminal "Leader Sequences"

Gene	Protein	Reference
ndh	Respiratory NADH dehydrogenase	Young *et al.*,(1981)
lacY	Lactose permease	Ehring, Beyreuther, Wright and Overath, (1980); Büchel, Gronenborn and Müller-Hill, (1980)
uncB *uncF* *uncE*	Proteins comprising the proton channel of ATP-synthase	Gay and Walker, (1981)
frdA	Flavoprotein subunit of the fumarate reductase	Cole, (1982)
frdB	Iron-sulphur subunit of fumarate reductase	Cole *et al.*, (1982)
malK	Component of the maltose transport system	Gilson *et al.*, (1982a)
hisP	Component of the histidine transport system of *S. typhimurium*	Higgins *et al.*, (1982)

Nonetheless, it must be stressed that the helical hairpin hypothesis awaits experimental verification.

Reconstitution of Membrane Proteins into Membrane Vesicles

In the past decade the reconstitution of purified membrane proteins into artificial bimolecular lipid membranes has become a powerful tool in understanding the various functions of membrane proteins especially those involving transport activity (Racker, 1979; Hokin, 1981; Eytan, 1982).

Membrane proteins have been reconstituted into artificial membranes by a variety of procedures including: (a) cholate (or other detergent)-dialysis, (b) detergent dilution, (c) sonication, (d) freeze-thaw-sonication and (e) incorporation and reconstitution into planar monomolecular and bimolecular membranes.

The cholate dialysis procedure has been widely used and will be the only method further discussed here. The basic procedure is to mix the purified protein with an excess of phospholipid and detergent, usually cholate, and often at high ionic strengths. The detergent is then removed by dialysis over a period of 1-2 days. During this period, liposomes containing the protein are formed (Hokin, 1981). One disadvantage of the cholate dialysis procedure is that prolonged exposure to detergent can lead to inactivation of some membrane proteins. Hence, each membrane protein responds differently to the various reconstitution procedures, and at present the approach is entirely empirical (Racker, 1979).

The orientation of membrane proteins in biological membranes is asymmetric (Rothman and Lenard, 1977). The proteins are confined to a specific surface of the membrane. Transmembranous proteins span

the membrane unidirectionally with a specific pole always protruding in the same direction. However, with reconstituted membrane proteins unidirectional orientation has been observed in some cases while in other cases the proteins are orientated in both directions across the membrane (Eytan, 1982).

Approximately a decade ago Kaback and his coworkers developed techniques for the preparation of bacterial membrane vesicles (Kaback, 1971; Kaback, 1974a,b). These vesicles have been characterized in detail (ibid.) and exhibit the same polarity and configuration as the membrane of the intact cell (Owen and Kaback, 1979a,b).

A number of different purified membrane-bound dehydrogenases have been reconstituted into prepared membrane vesicles. The following purified membrane-bound dehydrogenases when mixed with E. coli membrane vesicles can spontaneously insert into the membrane: the D-lactate dehydrogenase (Futai, 1974; Short, Kaback and Kohn, 1974; Halder, Olsiewski, Walsh, Kaczorowski, Bhaduri and Kaback, 1982), the glycerol-3-phosphate dehydrogenase (Futai, 1974; Schryvers, Lohmeier and Weiner, 1978), the D-amino acid dehydrogenase (Olsiewski, Kaczorowski, Walsh and Kaback, 1981; Halder et al., 1982), and the respiratory NADH dehydrogenase (Jaworowski et al., 1981b).

Interestingly, reconstitution of D-lactate-dependent transport and oxidase activity has been observed in membrane vesicles with purified D-lactate dehydrogenase bound to either surface of the membrane (Short et al., 1974; Halder et al., 1982). Similarly, reconstitution of both right-side-out and inside-out vesicles with D-amino acid dehydrogenase has been reported (Olsiewski et al., 1981;

Halder et al., 1982). Furthermore, in the presence of D-alanine, reconstituted right-side-out and inside-out vesicles generated electrochemical proton gradients of similar magnitude but opposite polarity, indicating that enzyme bound to either surface of the membrane is physiologically functional (Olsiewski et al., 1981).

It has been demonstrated that active NADH oxidase can be reconstituted from the pure NADH:ubiquinone oxidoreductase and membrane vesicles prepared from ndh mutant strains (Jaworowski et al., 1981b). Jaworowski et al. (1981b) demonstrated that the purified enzyme preparation is reconstitutively far less active after dialysis against low ionic buffer. The decreased ability to reconstitute ndh mutant membrane vesicles after dialysis against low ionic strength buffer is reversible, since a reconstitutively active preparation can be obtained by further dialysis of such reconstitutively inactive preparations against 1 M potassium phosphate buffer (ibid.). Therefore, it is unlikely that the loss of a component of the enzyme during dialysis leads to a lowered efficiency of reconstitution.

The ease with which the purified NADH dehydrogenase inserted into mutant membrane vesicles and the reversible effect of ionic strength on the efficiency of reconstitution provided an interesting basis for further study. In the work described in this chapter, the effect of ionic strength on the efficiency of reconstitution was examined in more detail and reconstitution experiments were performed with spheroplasts to determine whether the purified NADH dehydrogenase could insert into the outer surface of the E. coli inner membrane. Conditions were also established for

efficient reconstitution of the enzyme into phospholipid vesicles by the cholate dialysis procedure. Consideration of the membrane biology of the E. coli NADH dehydrogenase is hampered by the lack of information concerning the membrane sector of the enzyme. It is clear that a portion of the enzyme including the NADH binding site is accessible from the cytoplasm. However, the extent and disposition of the membrane sector of the enzyme is unknown. In an attempt to obtain further information concerning this point the approach of limited proteolysis was used and these experiments are also described in this Chapter.

Investigation of the Membrane Topography of Membrane Proteins by Proteolysis

Despite progress in different areas of structural research, crystallography is still the only way to determine the three dimensional structure of proteins to atomic resolution. Unfortunately, membrane proteins are inherently difficult to obtain in a suitable crystalline form. Only in the case of bacteriorhodopsin from the purple membrane of the Halobacterium (Michel and Oesterhelt, 1980), porin, an outer membrane protein from E. coli (Garavito and Rosenbusch, 1980) and the mitochondrial cytochrome bc_1 complex (Ozawa, Tanaka and Shimomura, 1983) has the crystalline structure been demonstrated by X-ray diffraction (review, Michel, 1983). However, these crystals have not been satisfactory in that they have not led to the determination of the proteins' three dimensional structure to atomic resolution.

For proteins that are anchored in the membrane by a short hydrophobic tail, the functional part can often be released intact

from the membrane by suitable treatment with protease(s). The part that is released can then be treated as a soluble protein. The soluble form of cytochrome $\underline{b}_5$ was crystallized about 10 years ago by Mathews, Levine and Argo (1972).

The aspect of cytochrome $\underline{b}_5$ and its reductase which has generated the greatest interest is their clearly amphipathic structure. Both consist of a globular N-terminal domain with a relatively polar amino acid composition containing the haem or the flavin group, and a smaller distinctly non-polar C-terminal domain. The domains can be separated and isolated because the linking regions are particularly susceptible to protease. Tryptic cleavage of the cytochrome $\underline{b}_5$ molecule yields a polar fragment, containing haem, a hydrophobic fragment associated with lipids and a linking peptide of 15 residues (Visser, Robinson and Tanford, 1975; Spatz and Strittmatter, 1971). Similarly, cleavage by chymotrypsin converts the 43000 mol wt NADH:cytochrome $\underline{b}_5$ reductase protein into a core enzyme (mol wt 33000) and aggregated hydrophobic peptides (Spatz and Strittmatter, 1971; 1973).

In some other enzyme systems limited proteolysis has also been used successfully as a tool to identify membrane protein topography. Some examples include bovine cytochrome $\underline{b}_5$ (Fleming, Dailey, Corcoran and Strittmatter, 1978), Torpedo acetylcholine receptor (Wennogle and Changeux, 1980; Strader and Raftery, 1980) and the rat renal brush-border membrane γ-glutamyltranspeptidase and aminopeptidase (Tsao and Curthoys, 1980). Indeed, proteolysis was an essential strategy in the elucidation of the structure of bacteriorhodopsin (Ovchinnikov, Abdulaev, Feigina, Kiselev and Lobanov, 1977; 1979).

With some enzymes, limited action of a particular proteolytic enzyme has led to the separation and purification of separate domains. This procedure was successful with the bifunctional Saccharomyces cerevisiae flavocytochrome b_2, in which the flavoprotein domain (carrying FMN) and cytochrome domain (with the haem) were able to be separately purified (Gervais and Tegoni, 1980). A similar approach was used with the NAD-specific glutamate dehydrogenase of Neurospora crassa which led to the elucidation of two distinct domains which possibly arose by the fusion of two distinct genes (Haberland, Chen and Smith, 1980).

Proteolysis studies of both the purified NADH dehydrogenase and genetically amplified IY91 membrane vesicles were undertaken with the aim of: (a) establishing the membrane topography of the respiratory NADH dehydrogenase, (b) 'liberating' any soluble portion of the enzyme from the membrane, (c) cleaving the enzyme at an interdomain site producing separate domains, one or more of which may possess partial catalytic activity and (d) defining the membrane-embedded amino acid sequence(s).

MATERIALS AND METHODS

Media

The mineral salts medium employed has been described elsewhere (Stroobant, Young and Gibson, 1972) and was used at normal strength. Glucose and other supplements were sterilized separately and were added to give the following concentrations: 30 mM glucose, 40 mM succinate, 0.1% Casamino acids, 0.15 mM L-histidine HCl, 0.3 mM

L-isoleucine, 0.3 mM L-valine, 0.2 mM L-tryptophan and 1 μM thiamine. The amino acids were obtained from Sigma Chemical Company (St. Louis, MO). Membranes were prepared as described in Chapter 3.

Bacterial Strains and Plasmids

The ndh mutant strain IY12 (thi his ilv trp rps ndh), a derivative of E. coli K12, has been described previously (Young et al., 1978). IY13 (thi his ilv trp rpsL) is an isogenic transformant of IY12.

Plasmid pIY1 (Young et al., 1978) possesses the 2500 base pair ndh fragment cloned into the EcoRI site of pSF2124 (So et al., 1975). Plasmid pLJ3 (Johnsrud, 1978) carries a double lac promotor fragment at the EcoRI site of pMB9 (Rodriguez, Bolivar, Goodman, Boyer and Betlach, 1976). Plasmid pIY10 is a derivative of pMB9, carrying the ndh and double lac promotor fragments at the EcoRI site. Plasmid pIY10 was transformed into strain IY12 as described previously (Jaworowski et al., 1981a). Selection of transformants carrying the ndh gene recloned into pMB9 was made on mannitol-minimal plates (Young and Wallace, 1976). Viable colonies were then tested for the presence of hybrid plasmids containing the lac fragment by plating onto mannitol-minimal plates containing X-gal (Jaworowski, et al., 1981a,b). Strains with multicopy plasmids carrying the lac promotor produce blue colonies on this medium (Johnsrud, 1978). Membrane vesicles prepared from three such clones were assayed for NADH:ubiquinone oxidoreductase activity and the clone with the highest specific activity was designated IY91.

Reconstitution of NADH Oxidase in Membranes Derived from Ndh IY12 Mutants

Two methods were used to reconstitute NADH oxidase in ndh mutant membrane vesicles using purified NADH:ubiquinone oxidoreductase.

In the first method, 50 µL aliquots of membrane vesicles were placed into Eppendorf tubes (1.5 mL volume) at 0°C, and varying amounts of pure enzyme (0-80 µL of purified enzyme in 0.65 M potassium phosphate buffer, pH 7.5, ~30 units mL^{-1}; or enzyme from pooled peaks of NADH:ubiquinone oxidoreductase activity recovered from sucrose density gradients, ~10 units mL^{-1}) were added and mixed thoroughly. 5 µL of the mixture was assayed for NADH oxidase in an assay mixture containing 1 mL 50 mM Tes buffer, pH 7.5, 20 µM FAD and 250 µM NADH, preincubated at 30°C. The reaction was initiated by the addition of reconstituted membrane vesicles and followed at 340 nm. The rate of NADH oxidation was calculated using $\Delta\varepsilon = 6220$ $M^{-1}cm^{-1}$.

In the second method, 5 µL of ndh mutant membrane vesicles or spheroplasts was placed into 1 mL of assay buffer in a 1 mL quartz cuvette and preincubated at 30°C. In the spheroplast reconstitution experiment the assays were done in STM buffer with 250 µM NADH present. In STM buffer the integrity of the spheroplasts was maintained. Various volumes of pure enzyme (0-80 µL of purified enzyme in 0.65 M potassium phosphate, pH 7.5, ~30 units mL^{-1}; or enzyme from pooled peaks of NADH:ubiquinone oxidoreductase activity obtained from sucrose density gradients, ~10 units mL^{-1}) were added and NADH oxidation was followed as described above.

The rates of NADH oxidation obtained in both cases were corrected for the NADH oxidase activities of the mutant membrane

vesicles and the pure enzyme, measured individually in the above assay system. The membrane vesicles referred to throughout this chapter have an inside-out orientation unless specified otherwise. The IY12 spheroplasts did not possess any NADH oxidase activity. The relationship between the NADH oxidase activity of the purified enzyme and added volume was linear over the range used in these experiments.

Preparation of Spheroplasts from E. coli K12 Strains

Spheroplasts from different strains of E. coli K12 were prepared essentially as described by Kaback, (1971). Cells were grown in minimal media plus supplements until late logarithmic phase (~180 Klett units; Klett-Summerson colorimeter fitted with a blue filter). Cells were harvested by centrifugation (6000 rpm, 10 minutes, GSA Sorvall rotor) and the wet weight was determined.

10 g wet weight of cells were resuspended in 200 mL of 10 mM Tris HCl, pH 7.5, at 0°C. The cells were pelleted and resuspended again in the same volume of fresh buffer. The cells were then repelleted and resuspended in 100 mL STM buffer. The cells were repelleted and resuspended in the same volume of fresh STM buffer. Potassium EDTA, pH 7.0, and lysozyme (egg white, Sigma Chemical Co.) were added to give final concentrations of 10 mM and 0.5 mg mL^{-1}, respectively. The cells were slowly stirred for 30 minutes at room temperature. The spheroplasts were pelleted, and resuspended in 100 mL of STM buffer and then pelleted once again. The spheroplasts were then resuspended in a minimum volume of STM buffer (~20 mL). The volume of the spheroplast suspension was adjusted so that 10 µL of spheroplasts added to 1 mL of STM buffer gave an absorbance of 0.2 units at a wavelength of 600 nm.

Reconstitution of Purified NADH:ubiquinone Oxidoreductase into Soybean Phospholipid Vesicles

The procedure used to reconstitute the purified enzyme was as described below: 80 mg of acetone/ether extracted (ie neutral lipid free) phospholipids from soybean were dissolved in 2 mL $CHCl_3$, dried under a stream of N_2 and then dissolved in 2 mL diethyl ether. 2 mL of cholate buffer (1.0% (w/v) cholate, 5 mM potassium phosphate, pH 7.5, 1 mM EDTA, 1 mM 2-mercaptoethanol and 20 μM FAD) was added to the dried phospholipid and sonicated (3x1 minutes at 0°C) until homogeneous. 1 mg of concentrated enzyme was added to the phospholipid mix and left at 0°C for 5 minutes. The enzyme had previously been extensively dialyzed against 5 mM potassium phosphate, pH 7.5, 0.1% cholate (w/v), 20 μM FAD, 1 mM EDTA and 1 mM 2-mercaptoethanol.

The enzyme-phospholipid mix was placed into a dialysis bag and extensively dialyzed against a buffer composed of 5 mM potassium phosphate, pH 7.5, 1 mM EDTA, 1 mM 2-mercaptoethanol and 20 μM FAD at 4°C. The dialyzed mixture was then diluted with dialysis buffer to 3.8 mL volume and the phospholipid vesicles were pelleted by ultracentrifugation (38000 rpm, 3 hours, SW56 Beckman rotor). The pellet was resuspended to 1 mL in dialysis buffer at 4°C.

Sucrose Density Gradients

Stock sucrose solutions (BDH chemicals, analytical reagent) of 50% (w/v) sucrose in distilled water and 0.5 M and 0.7 M potassium phosphate buffer, pH 7.5, were prepared. A two compartment perspex gradient mixer was used to pour a 10 mL volume 5-30% (w/v) linear sucrose gradient in an 11 mL capacity SW41 Beckman polyallomer

centrifuge tube. In all cases, 50 µL of concentrated purified enzyme (5.2 mg mL^{-1} protein) after dialysis against either 5 mM potassium phosphate, pH 7.5, or 0.65 M potassium phosphate, pH 7.5, (also including 0.1% (w/v) cholate, 20 µM FAD, 1 mM EDTA and 1 mM 2-mercaptoethanol) was loaded onto the top of the gradient. The gradient was centrifuged for 14.5 hours, 40000 rpm at 0°C in an SW41 Beckman centrifuge. After centrifugation ~80 fractions of ~120 µL volume were collected in sterile plastic trays by piercing the bottom of the tube with a syringe needle. Fractions were assayed immediately after collection. The peaks of NADH:ubiquinone oxidoreductase activity were pooled and snap frozen using liquid nitrogen.

Both the high and low ionic strength gradients were calibrated with standard globular protein markers. The markers used were *E. coli* β-galactosidase (Worthington Biochemical Corporation, New Jersey), mol wt ~540000, $S_{20,w}$ = 13.9, bovine liver catalase (Sigma Chemical Co., St. Louis), mol wt ~225000, $S_{20,w}$ = 11.3 and human haemoglobin, mol wt ~57000, $S_{20,w}$ = 4.3. The human haemoglobin was purified from packed red blood cells according to the following procedure. Cells were washed in 0.9% (w/v) NaCl and lyzed by shaking with two volumes of cold (0°C) distilled water and 0.5 volume of CCl_4. Debris was pelleted by centrifugation for 5 minutes in a bench MSE centrifuge. Sodium phosphate buffer, pH 6.0, was added to the supernatant to give a final concentration of 0.1 M. The solution was then recentrifuged and the supernatant removed. After purification, 25 mL of a 50 mg mL^{-1} solution was obtained. 0.5 mL aliquots were dialyzed against 5 mM and 0.7 mM potassium phosphate buffer, pH 7.5, containing 0.1% (w/v) cholate. 5 µL of the dialyzed sample

was diluted to 50 µL with dialysis buffer and then loaded onto the top of the sucrose gradients. Stock solutions of β-galactosidase (5 mg mL^{-1}) and catalase (5 mg mL^{-1}) were dialyzed as above and 50 µL of each was added undiluted to the top of the sucrose gradients.

Each standard protein was detected by measuring the absorbance at a wavelength of 405 nm of 50 µL of the fractions added to 1 mL of 50 mM potassium phosphate buffer, pH 7.5. Assays of catalase and β-galactosidase were also performed. 5 µL of fractions from the catalase peaks, detected by absorbance at 405 nm, were added to a 1 mL quartz cuvette containing 1 mL of 50 mM potassium phosphate buffer, pH 7.5, plus 20 mM H_2O_2 and the change in absorbance at a wavelength of 240 nm was recorded. β-galactosidase was assayed by the addition of 5 µL from gradient fractions to a 1 mL assay mixture containing 0.50 mg mL^{-1} of o-nitrophenyl-β-D-galactopyranoside in 50 mM potassium phosphate, pH 7.5 buffer. The change in absorbance at a wavelength of 405 nm was recorded.

Proteolysis of Purified Enzyme and Membrane Vesicles

Trypsin (bovine) which had been treated with TPCK (L-(tosyl amido-2-phenyl) ethyl chloromethyl ketone) to inhibit contaminating chymotryptic activity, was purchased from Worthington Biochemical Corporation, Freehold New Jersey. Chymotrypsin (bovine) was also from Worthington Biochemical Corporation, New Jersey and subtilisin A and pronase CB (B grade) were purchased from Novo Industries Copenhagen. Stock solutions of 4 mg mL^{-1} in 5 mM potassium phosphate buffer, pH 7.5, of the appropriate proteolytic enzyme were freshly prepared prior to each experiment. Protease inhibitors were not added to the proteolysis mixture unless otherwise stated. After

incubation with proteolytic enzyme (2% w/w protein), samples were removed and derivatized in boiling (100°C) SDS sample buffer (30 mM Tris HCl, pH 6.8, 5% (w/v) SDS, 5% (v/v) 2-mercaptoethanol and 5% (w/v) glycerol) for 5 minutes. Therefore, samples were denatured as quickly as possible to minimize proteolysis during derivatization. Comparison of samples SDS derivatized in the presence (0 minutes incubation) and absence of protease demonstrated that significant proteolysis of the sample did not occur during derivatization (see Figure 4-8).

SDS-Polyacrylamide Gel Electrophoresis

SDS-polyacrylamide gel electrophoresis was performed with vertical slab gels (10 x 14 cm) in which the separation gel contained a linear 10-25% (w/v) concentration gradient of acrylamide. The gels also possessed a stacking gel of 4.5% (w/v) polyacrylamide, using an apparatus similar to that described by Studier (1973). The discontinuous SDS buffer system of Laemmli (1970) was used. Gels were run at a constant current of 30 mA for 3-4 hours at room temperature. Gels were fixed at 65°C for 20 minutes in 200 mL of 10% (w/v) trichloroacetic acid and stained with 200 mL Coomassie Brilliant Blue R250 (0.1% (w/v) in destainer) for 20 minutes at 65°C then destained at room temperature using a mixture of ethanol/glacial acetic acid/water (25:8:67 v/v/v).

Gels were scanned using a Schoeffel model SD3000 spectrodensitometer at 540 nm and using a slit width of 0.5 mm and the relative amounts of polypeptides were estimated. For the determination of molecular weights the following proteins were used as standards: ovalbumin, catalase, lysozyme, myoglobin, cytochrome c and human haemoglobin (95% pure).

The amounts of protein loaded onto the SDS-polyacrylamide gels were ~10 µg protein per lane for the purified enzyme and ~400 µg protein per lane for the membrane vesicles. Protein concentrations were determined as described in Chapter 3.

Sephadex G-75 Chromatography of Purified NADH:ubiquinone Oxidoreductase

0.5 mL of concentrated enzyme (3.1 mg mL^{-1}, 204 units mg^{-1} protein) was chromatographed on a Sephadex G-75 column (80 mL total volume). The column was equilibrated with a buffer composed of the following: 0.65 M potassium phosphate, pH 7.5, 0.1% (w/v) cholate, 1 mM EDTA and 1 mM 2-mercaptoethanol. Chromatography was performed at 4°C. Fifty fractions, each of 0.86 mL volume, were collected and assayed for NADH:ubiquinone and NADH:$Fe(CN)_6^{3-}$ oxidoreductase activities as described in Chapter 3.

Purified enzyme was digested with trypsin and chromatographed as described below: 0.7 mL of concentrated enzyme (3.1 mg mL^{-1}, 204 units mg^{-1} protein) was incubated with 15 µL of a 4 mg mL^{-1} solution of trypsin for 2.5 hours at 25°C. 25 µL of an ethanolic solution of phenylmethylsulphonyl fluoride (PMSF) with a concentration of 20 mg mL^{-1} was added and the mixture was incubated for a further 45 minutes. After incubation, 0.5 mL of the enzyme solution was chromatographed on the G-75 Sephadex column as described above and the remaining 0.2 mL was dialyzed at 4°C for 16 hours against a buffer composed of 5 mM potassium phosphate, pH 7.5, 0.1% (w/v) cholate. 5 µL of the dialyzed sample was derivatized in sample buffer and subjected to electrophoresis on an SDS-polyacrylamide gel.

RESULTS AND DISCUSSION

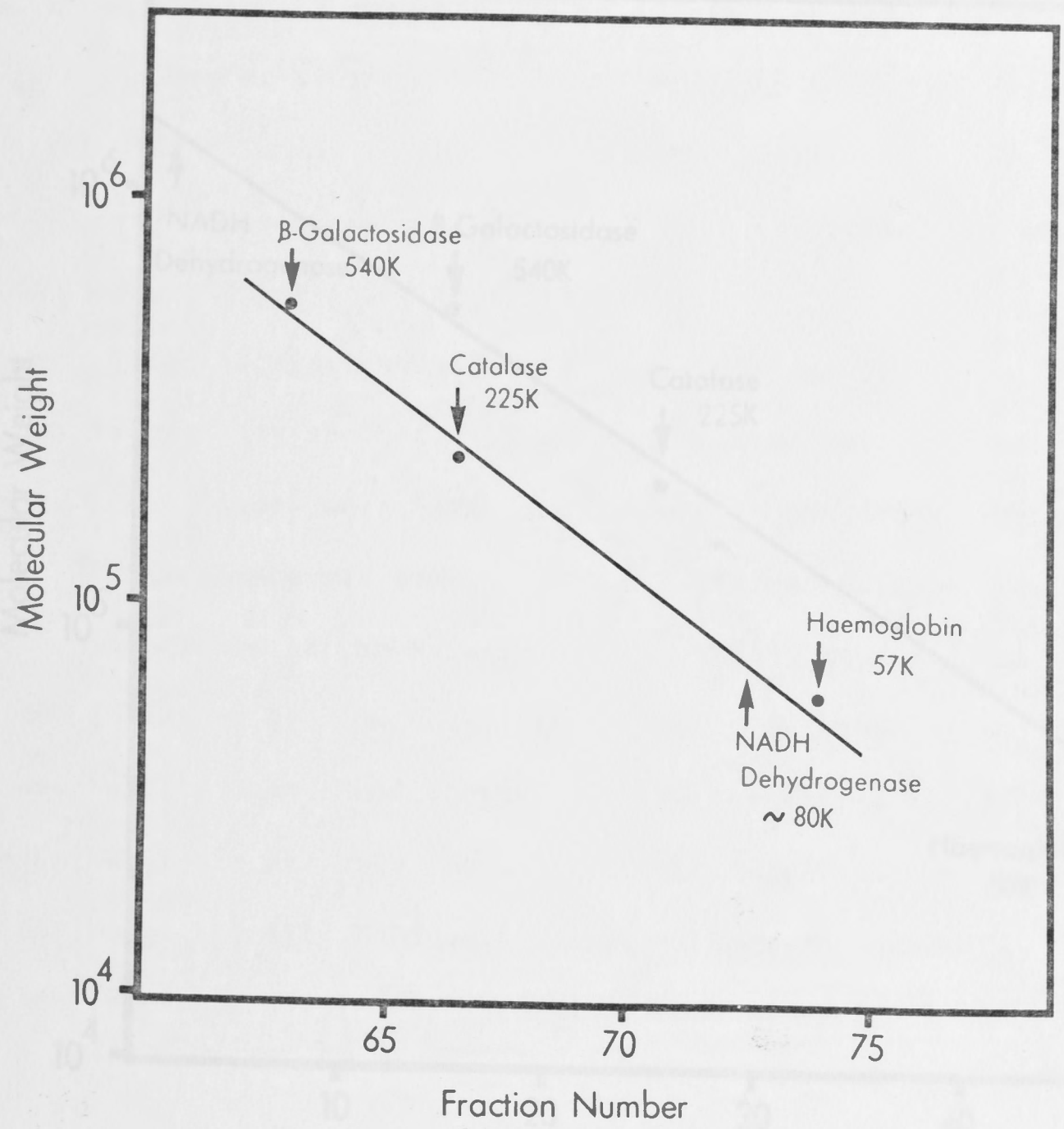
Correlation Between Aggregation State, Ionic Strength and Reconstitutive Activity

The purified NADH dehydrogenase has been shown to spontaneously insert into membranes isolated from ndh strains of E. coli and to reconstitute the NADH oxidase activity (Jaworowski et al., 1981b). This interesting property has been reported to be largely abolished if the purified enzyme is dialyzed against low ionic strength buffer (ibid.). The state of aggregation of the purified enzyme in response to ionic strength has been examined as a possible reason for the altered efficiency of reconstitution.

Samples of purified NADH:ubiquinone oxidoreductase were dialyzed against different ionic strength buffers each having 0.1% (w/v) cholate present. Dialyzed enzyme samples were loaded onto 5-30% (w/v) linear sucrose gradients and ultracentrifuged under conditions described in the Materials and Methods section. Purified NADH:ubiquinone oxidoreductase and internal globular proteins were subjected to sucrose density gradient ultracentrifugation in the same gradient tube. The gradient profiles in high and low ionic strength buffers are shown in Figures 4-1 and 4-2, respectively. The enzyme activity peaks were as expected and the recovery of enzyme activity was high, being 40% for the 0.7 M potassium phosphate gradient and 70% for the 3.5 mM potassium phosphate gradient.

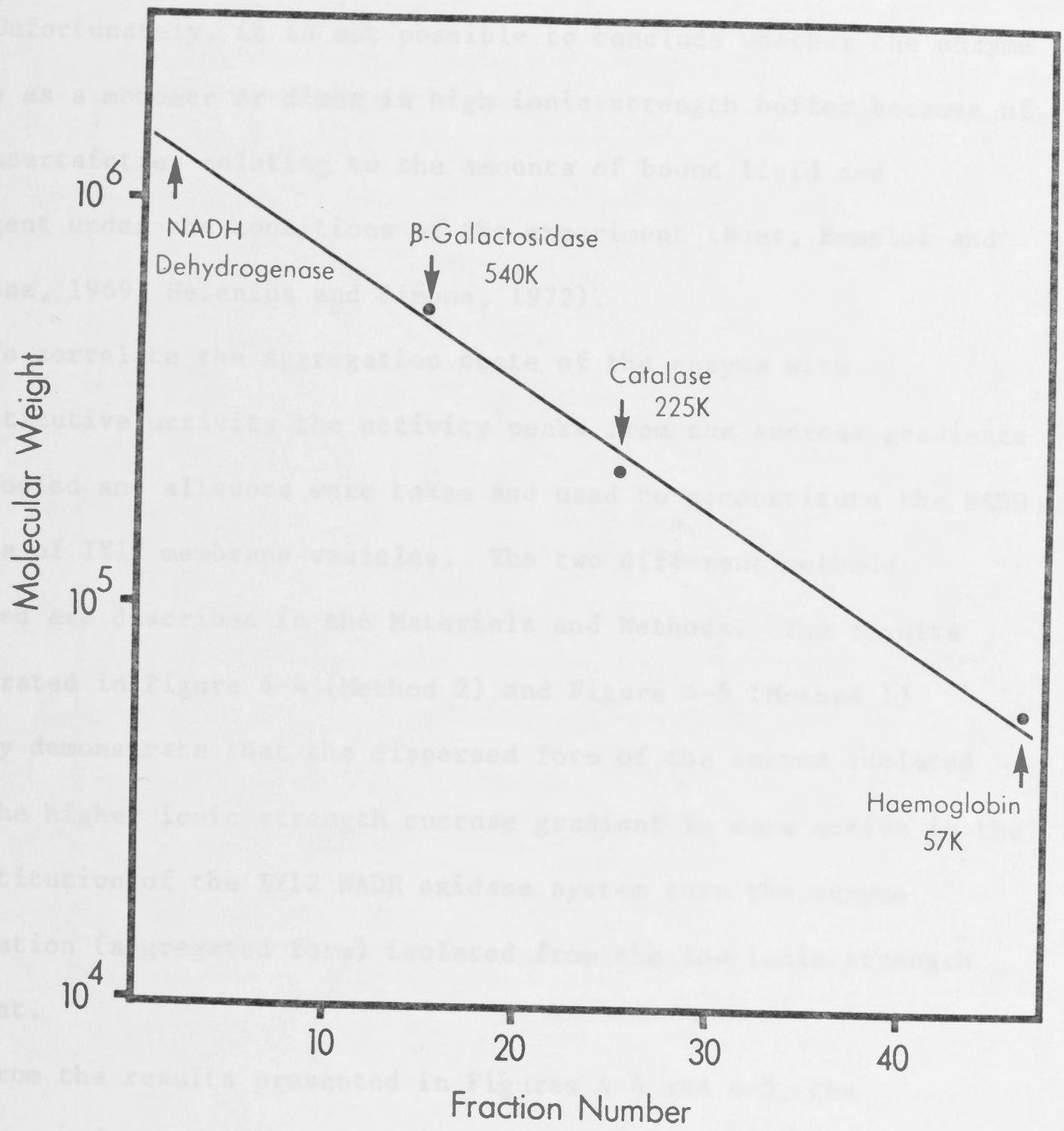
The gradient profiles demonstrate that the purified enzyme in low ionic strength buffer migrates through the sucrose gradient as a high molecular weight species. In contrast, the purified enzyme when centrifuged in high ionic strength buffer migrates through the

Figure 4-1. Sucrose Density Gradient Centrifugation of NADH:ubiquinone Oxidoreductase in High Ionic Strength Buffer



The purified enzyme was loaded onto a 5-30% (w/v) sucrose density gradient in 0.7 M potassium phosphate, pH 7.5 buffer, containing 0.1% (w/v) cholate. See Materials and Methods for further details.

Figure 4-2. Sucrose Density Gradient Centrifugation of NADH:ubiquinone Oxidoreductase in Low Ionic Strength Buffer



The purified enzyme was loaded onto a 5-30% (w/v) sucrose density gradient in 3.5 mM potassium phosphate, pH 7.5 buffer, containing 0.1% (w/v) cholate. See Materials and Methods for further details.

sucrose gradient with an M_r of 80000 and an estimated $S_{20,w}$ of 6.3 (Figure 4-1 and 4-3).

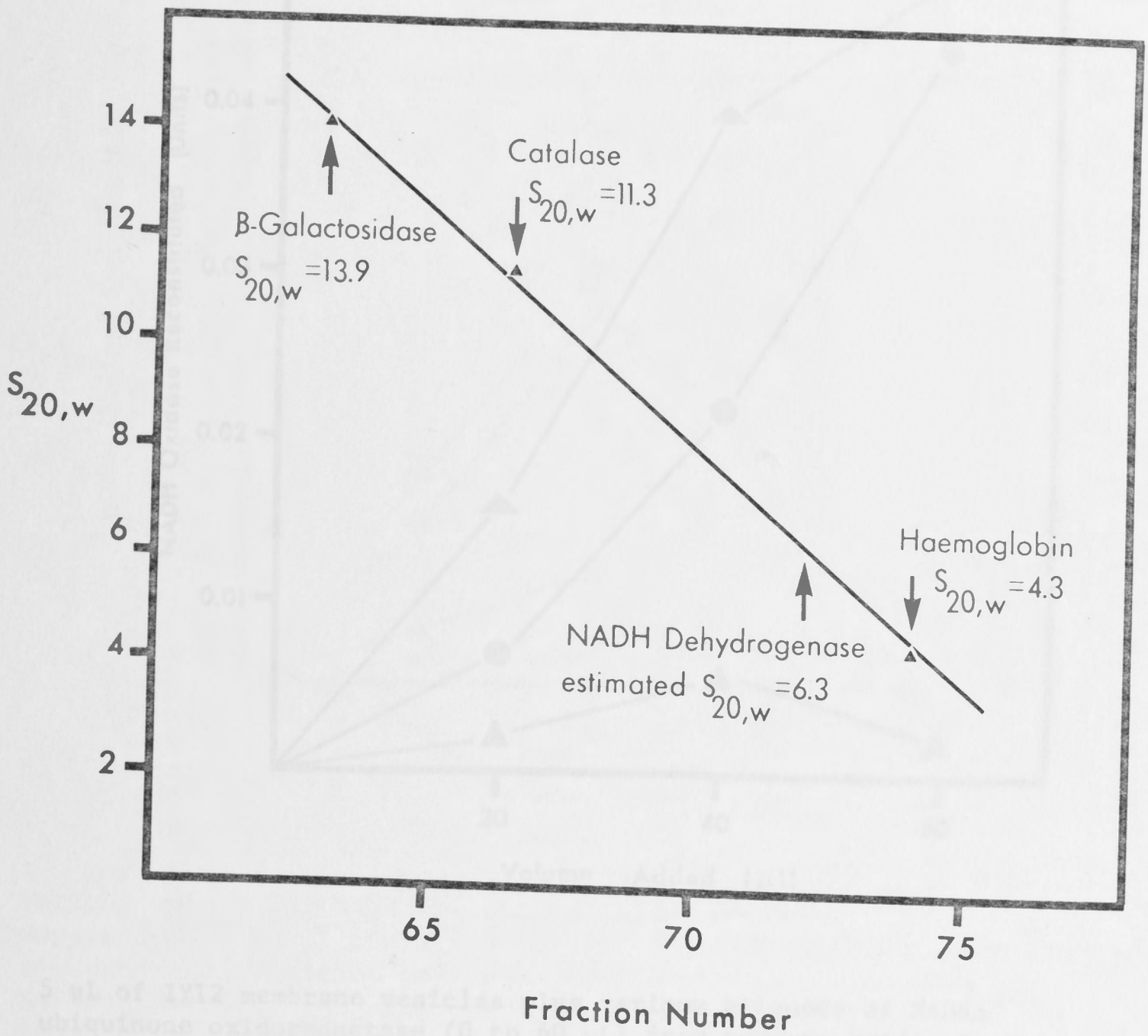
Unfortunately, it is not possible to conclude whether the enzyme exists as a monomer or dimer in high ionic strength buffer because of the uncertainties relating to the amounts of bound lipid and detergent under the conditions of the experiment (Bont, Emmelot and Vaz Diaz, 1969; Helenius and Simons, 1972).

To correlate the aggregation state of the enzyme with reconstitutive activity the activity peaks from the sucrose gradients were pooled and aliquots were taken and used to reconstitute the NADH oxidase of IY12 membrane vesicles. The two different methods employed are described in the Materials and Methods. The results illustrated in Figure 4-4 (Method 2) and Figure 4-5 (Method 1) clearly demonstrate that the dispersed form of the enzyme isolated from the higher ionic strength sucrose gradient is more active in the reconstitution of the IY12 NADH oxidase system than the enzyme preparation (aggregated form) isolated from the low ionic strength gradient.

From the results presented in Figures 4-4 and 4-5, the efficiency of reconstitution can be estimated. This is defined as the units of reconstituted NADH oxidase per unit of added NADH:ubiquinone oxidoreductase. In Figure 4-4, the efficiencies of reconstitution were: 3.5 mM gradient, 2.3%, 0.5 M gradient, 7.9%, and 0.7 M gradient, 16.9%. A similar trend was observed with the results presented in Figure 4-5.

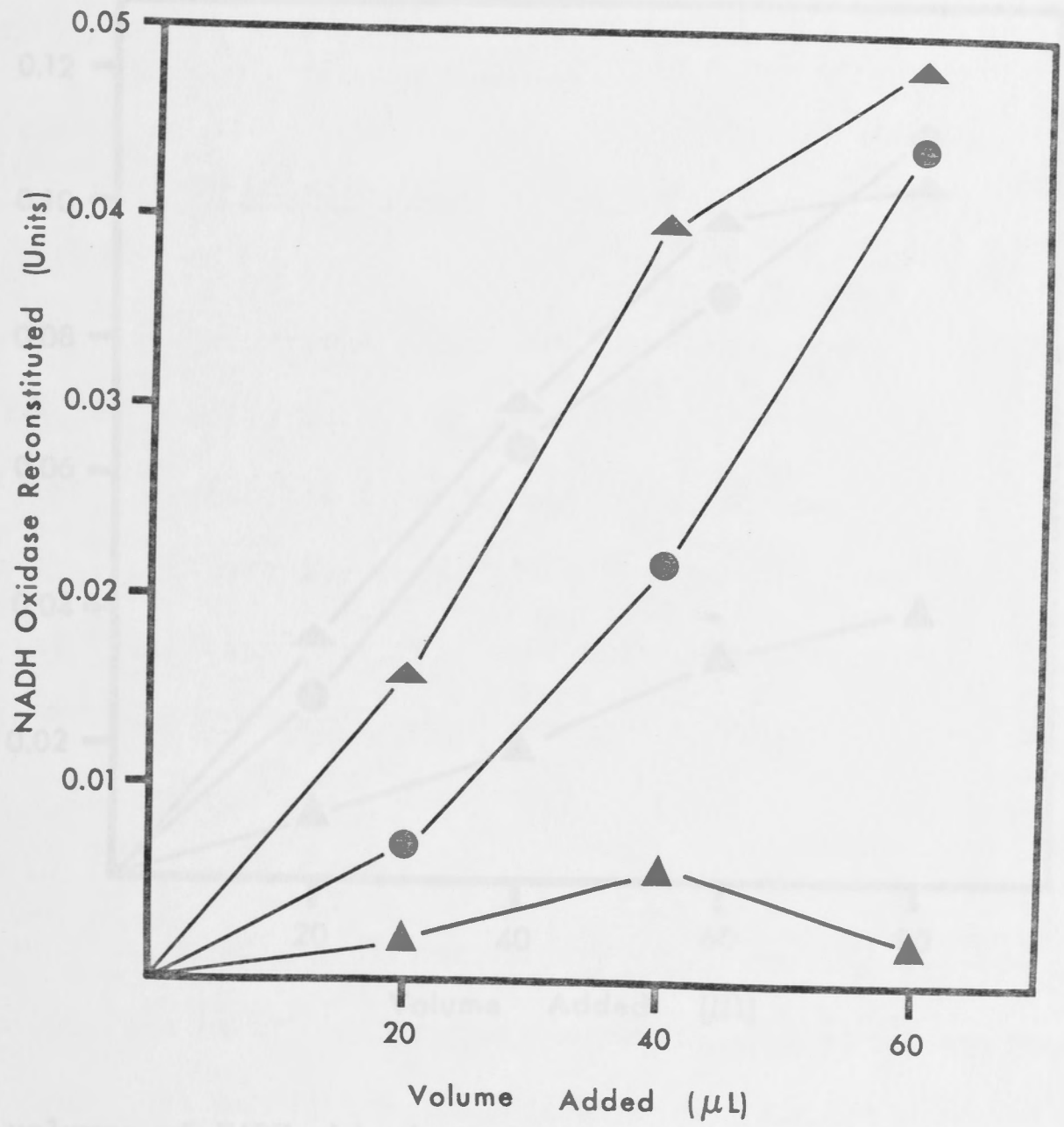
The apparent ease with which the dispersed form of the enzyme becomes membrane-associated suggests that such a mechanism could

Figure 4-3. Estimated $S_{20,w}$ Value for NADH:ubiquinone Oxidoreductase in High Ionic Strength Buffer



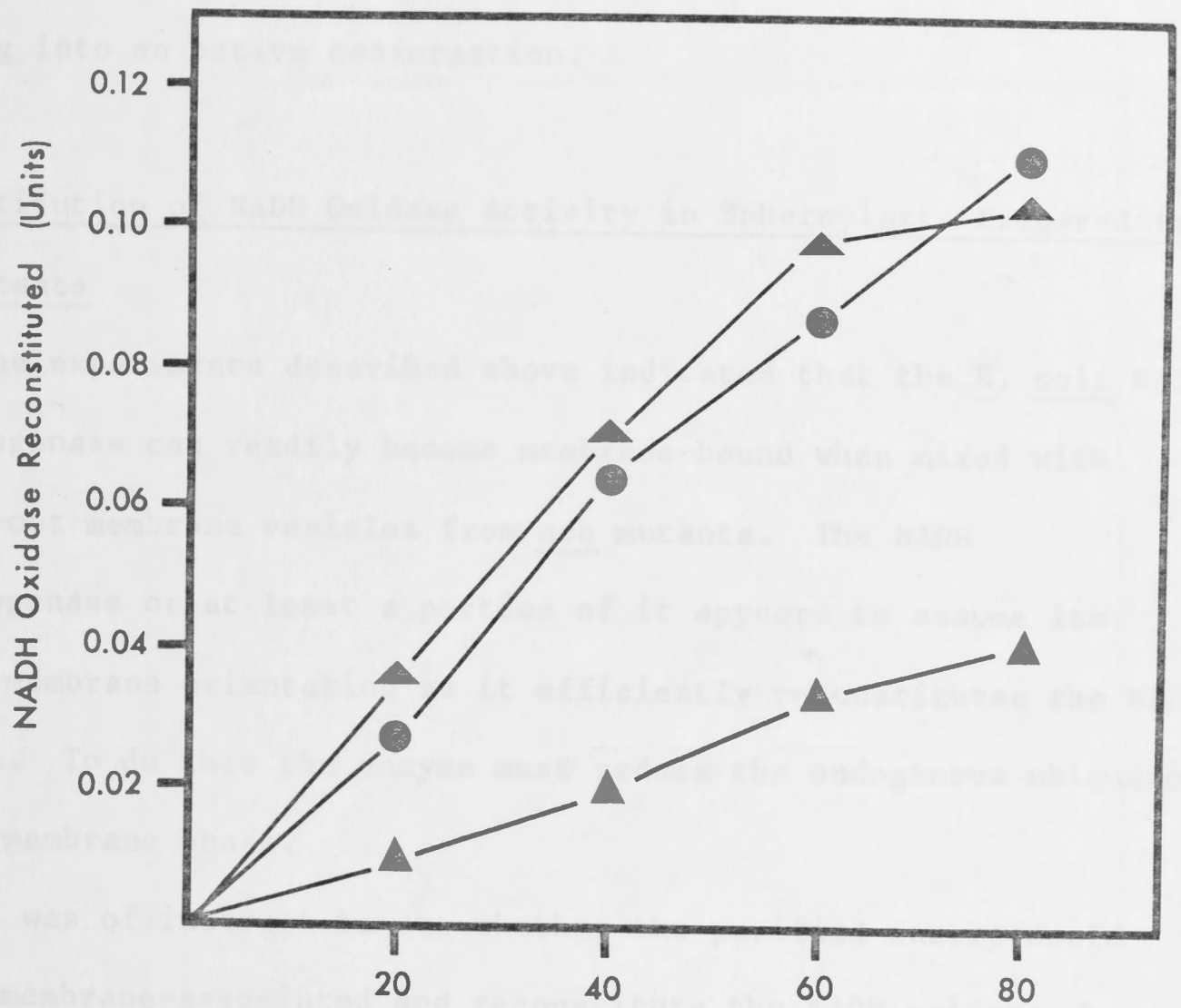
See Figure 4-1 and Materials and Methods for further details.

Figure 4-4. Reconstitution of NADH Oxidase in IY12 Membrane Vesicles: Addition of Enzyme Peaks from Sucrose Gradients



5 μL of IY12 membrane vesicles plus various aliquots of NADH: ubiquinone oxidoreductase (0 to 60 μL) from sucrose gradients were mixed together in 1 mL assay buffer and assayed spectrophotometrically for NADH oxidase. The enzyme samples added were from the following: (▲) 3.5 mM (10.1 units mL^{-1}), (●) 0.5 M (4.3 units mL^{-1}), and (▲) 0.7 M (10.3 units mL^{-1}) potassium phosphate sucrose gradients. The resulting activity was corrected for the NADH oxidase activity of the membrane vesicles (0.27 units mL^{-1}) and the pure enzyme samples (0.21, 0.15 and 0.26 units mL^{-1}, respectively). See Materials and Methods for further details.

Figure 4-5. Reconstitution of NADH Oxidase in IY12 Membrane Vesicles: Addition of Enzyme Peaks from Sucrose Gradients



Various volumes of NADH:ubiquinone oxidoreductase (0 to 80 μL) were added to 50 μL of IY12 membrane vesicles (at 0°C). The reconstituted vesicles were assayed spectrophotometrically for NADH oxidase and the rate corrected for the activity of the pure enzyme and IY12 membrane vesicles. The samples added were from the following: (▲) 3.5 mM (10.1 units mL^{-1}), (●) 0.5 M (4.3 units mL^{-1}), and (▲) 0.7 M (10.3 units mL^{-1}) potassium phosphate sucrose gradients. See Figure 4-4 and Materials and Methods for further details.

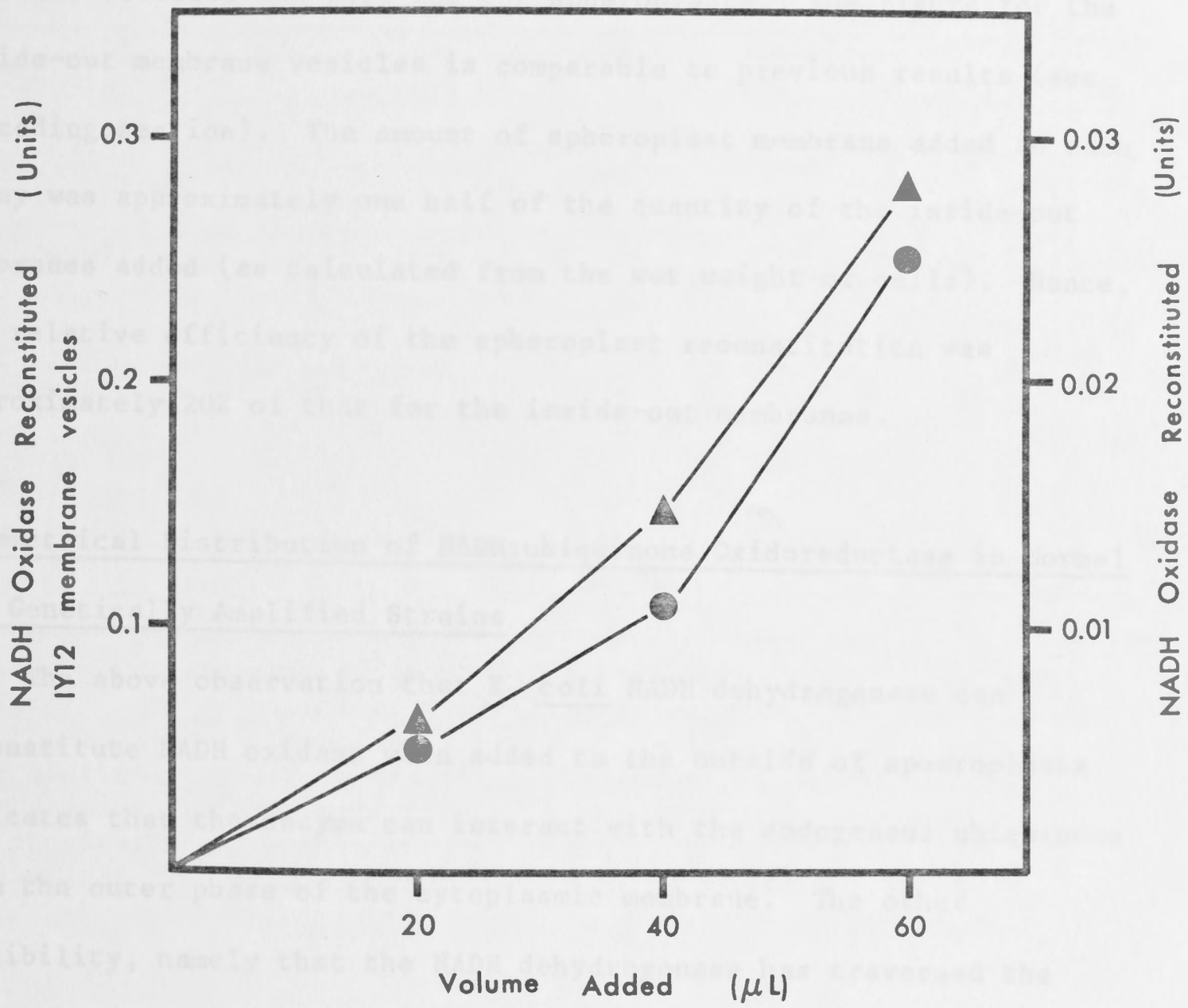
operate *in vivo*. The NADH dehydrogenase synthesized on membrane-bound ribosomes could associate or insert into the membrane after folding into an active conformation.

Reconstitution of NADH Oxidase Activity in Spheroplasts Prepared from *Ndh* Mutants

The experiments described above indicated that the *E. coli* NADH dehydrogenase can readily become membrane-bound when mixed with inside-out membrane vesicles from *ndh* mutants. The NADH dehydrogenase or at least a portion of it appears to assume its normal membrane orientation as it efficiently reconstitutes the NADH oxidase. To do this the enzyme must reduce the endogenous ubiquinone in the membrane phase.

It was of interest to see whether the purified enzyme could become membrane-associated and reconstitute the NADH oxidase when added to the outside of the cytoplasmic membrane. Spheroplasts were prepared from the *ndh* mutant strain IY12 according to the procedure described in the Materials and Methods section. Attempts were made to reconstitute the NADH oxidase in the spheroplasts using purified enzyme. A control experiment was carried out at the same time with inside-out membrane vesicles (prepared as described in Chapter 3). It is clear from the results shown in Figure 4-6 that purified enzyme can become membrane-associated when added to the outside of spheroplasts and reconstitute the NADH oxidase. This would suggest that reconstitution is not absolutely dependent on some specific mechanism operating from the cytoplasmic face of the inner membrane. The purified D-lactate dehydrogenase and D-amino acid dehydrogenase have also been reported to bind to either surface of the membrane (Short *et al.*, 1974; Olsiewski *et al.*, 1981; Halder *et al.*, 1982).

Figure 4-6. Reconstitution of NADH Oxidase in IY12 Membrane Vesicles and IY12 Spheroplasts



5 μL of either IY12 membrane vesicles (●) or 5 μL of IY12 spheroplasts (▲), plus various aliquots of NADH:ubiquinone oxidoreductase (0 to 60 μL; 22 units mL^{-1}) were mixed together in 1 mL assay buffer and assayed spectrophotometrically for NADH oxidase. The resulting activity was corrected for the NADH oxidase activity of the membrane vesicles (0.31 units mL^{-1}) and purified enzyme (0.36 units mL^{-1}). The spheroplasts alone did not possess any NADH oxidase activity.

The efficiency of reconstitution of each preparation represented in Figure 4-6 was calculated and found to be 19% for the inside-out membrane vesicles and 2.0% for the spheroplasts. The figure for the inside-out membrane vesicles is comparable to previous results (see preceding section). The amount of spheroplast membrane added in each assay was approximately one half of the quantity of the inside-out membranes added (as calculated from the wet weight of cells). Hence, the relative efficiency of the spheroplast reconstitution was approximately 20% of that for the inside-out membranes.

Asymmetrical Distribution of NADH:ubiquinone Oxidoreductase in Normal and Genetically Amplified Strains

The above observation that E. coli NADH dehydrogenase can reconstitute NADH oxidase when added to the outside of spheroplasts indicates that the enzyme can interact with the endogenous ubiquinone from the outer phase of the cytoplasmic membrane. The other possibility, namely that the NADH dehydrogenase has traversed the membrane and assumed its normal orientation is precluded by the impermeability of NADH. Only the externally orientated enzyme would be expected to transfer electrons from NADH to ubiquinone under these conditions. This being the case, one can obtain an estimate of the amount of enzyme oriented to the inside and to the outside in normal and genetically amplified strains from the levels of NADH oxidase and NADH:ubiquinone oxidoreductase in spheroplasts and membrane vesicles.

Comparison of the NADH oxidase levels in approximately equal quantities of spheroplasts and membrane vesicles from the wild type strain IY13 (Table 4-2) shows very little cyanide-sensitive NADH

Table 4-2. NADH Oxidase and NADH:ubiquinone Oxidoreductase Activities of Membrane Vesicles and Spheroplast Preparations

Preparation	NADH Oxidase (units mL^{-1})	NADH:Q-1 Oxidoreductase
Spheroplasts		
IY13	0.18	0.22
+KCN	0.16	
IY91	4.8	18.2
+KCN	0.4	
Membrane Vesicles		
IY13	5.7	13.3
+KCN	0.45	
IY91	30	900
+KCN	1.5	

Assays were performed as described in the Materials and Methods of Chapter 3. 5 µL volumes of each preparation were used per assay. In the case of the inside-out membrane vesicles this constituted the addition of ~200 µg protein. The amount of spheroplast membranes added was estimated to be approximately equal to that of the inside-out membranes (calculated from the quantity of wet weight of cells).

oxidase in spheroplasts. Similarly, very little NADH:ubiquinone-1 oxidoreductase activity (<2%) is detectable in spheroplasts of IY13 compared with membrane vesicles. This indicates that the vast majority of the enzyme is present with an orientation into the cytoplasm. In the case of IY91, where the NADH dehydrogenase is amplified ~70-fold, the same situation applies.

The maintenance of the asymmetrical distribution of the NADH dehydrogenase is expected since 'flipping' would involve movement of the hydrophilic regions of the enzyme through the membrane and this would be energetically very unfavourable.

Reconstitution of the Purified NADH:ubiquinone Oxidoreductase into Artificial Phospholipid Vesicles

The procedure used to reconstitute the purified enzyme was basically the cholate dialysis procedure described by Racker (1979) with minor modifications (see Introduction and Materials and Methods). The procedure is fairly efficient as shown in Figure 4-7. Only 40% of the enzymic activity is lost in the high (1.0% w/v) concentration of cholate.

The flow diagram illustrated in Figure 4-7 is for the reconstitution of purified enzyme in low ionic strength (5 mM potassium phosphate, see Materials and Methods). The same procedure was attempted in 0.65 M potassium phosphate buffer, pH 7.5, but it appeared that vesicles did not form.

Reconstitution of the enzyme into artificial phospholipid vesicles allows examination of the properties of the NADH dehydrogenase in a phospholipid environment in the absence of other respiratory proteins. Such vesicles were used to study electron

Figure 4-7. Flow Diagram for the Reconstitution of NADH:ubiquinone Oxidoreductase with Soybean Phospholipid

(1) Purified Enzyme
52 units mg^{-1} — Recovery of Activity 100%

+ Soybean phospholipid
+ 1% (w/v) Cholate (final)

(2) Dialysis

(3) Ultracentrifugation — 58%

(4) Resuspension of Pellet — 36%

Experimental details are described in the Materials and Methods section.

transfer to long chain isoprenologues of ubiquinone (Chapter 3). This procedure was also used in preliminary experiments in this laboratory aimed at testing whether the enzyme could function as a proton pump. To date no such activity has been detected (Campbell and Jaworowski, unpublished results).

Proteolysis as an Approach to Determining the Membrane Topography of the NADH:ubiquinone Oxidoreductase

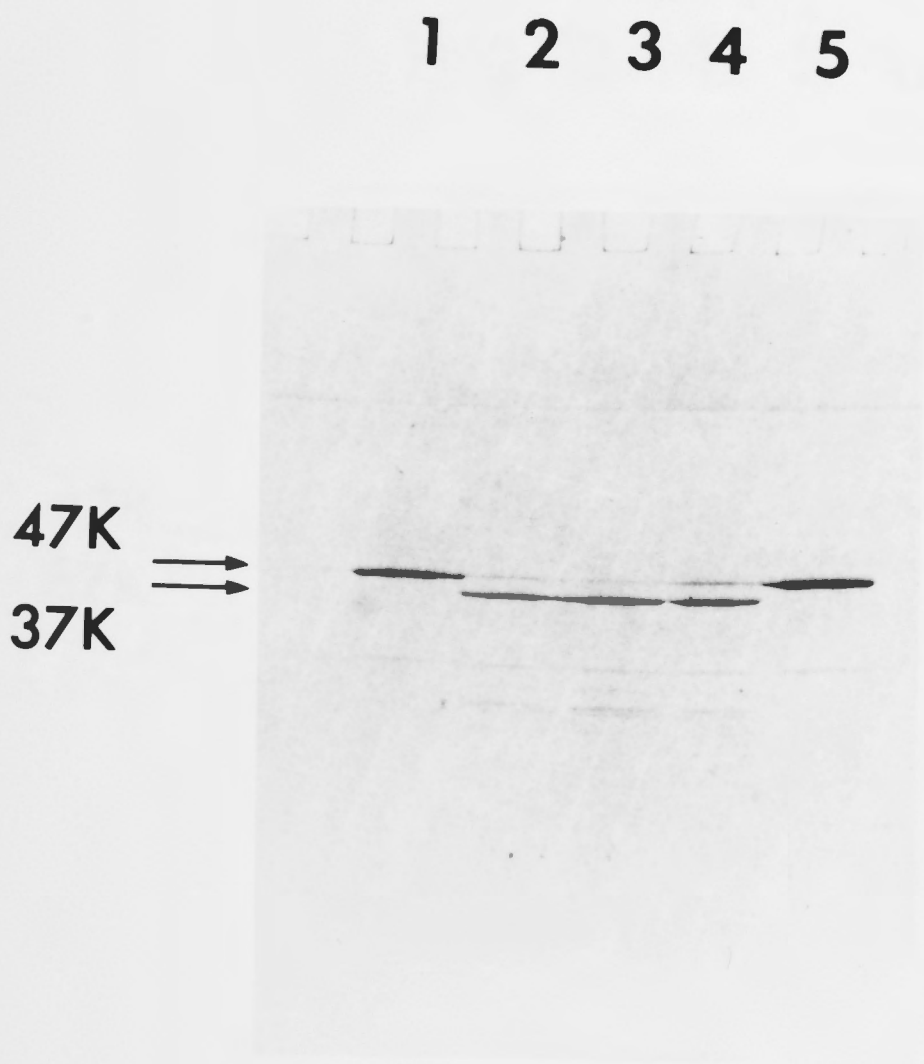
A number of different proteolytic enzymes were used in these studies, each having different substrate specificities. Trypsin hydrolyzes proteins at the carboxyl end of arginine and lysine residues and chymotrypsin at the carboxyl group of aromatic amino acid residues. Pronase and subtilisin both have broad specificities.

Proteolytic cleavage of the purified NADH:ubiquinone oxidoreductase by trypsin, chymotrypsin and pronase leads to the production of one dominant cleavage product of M_r ~37000 (see Figures 4-8, 4-9 and 4-10). It should be noted however that molecular weights of proteins derived from migration in SDS-polyacrylamide gels are not considered more accurate than ±10%.

The results presented in Figures 4-8, 4-9 and 4-10 were reminiscent of the membrane proteins, cytochrome $\underline{b}_5$ and NADH:cytochrome $\underline{b}_5$ reductase. By analogy with these proteins it was thought that proteolysis may result in the formation of fragments that separately retain their original functional properties.

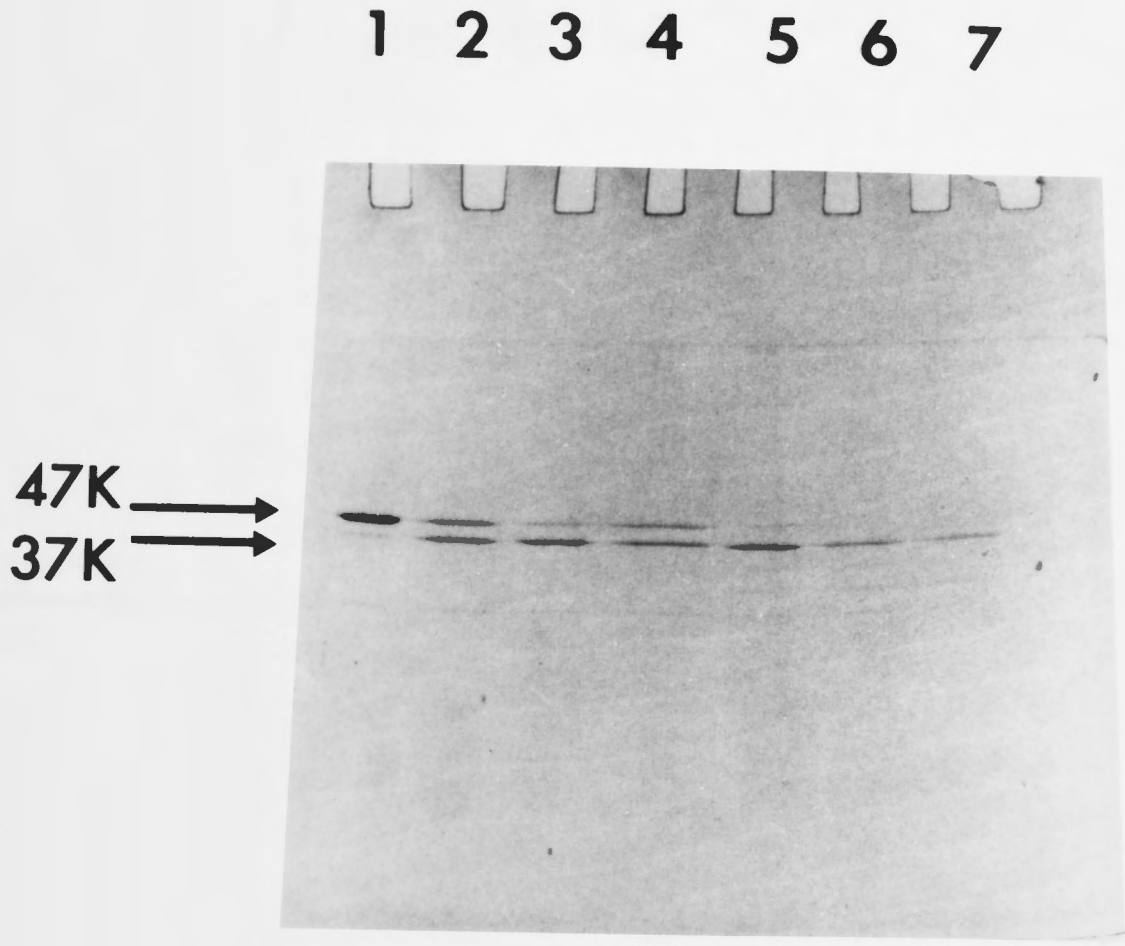
Samples of the trypsin and chymotrypsin digested enzyme (from the experiment illustrated in Figure 4-9) were snap frozen in liquid nitrogen at the same times that samples were removed for SDS

Figure 4-8. SDS-Polyacrylamide Gel Electrophoresis of NADH:ubiquinone Oxidoreductase after Proteolytic Digestion with Trypsin



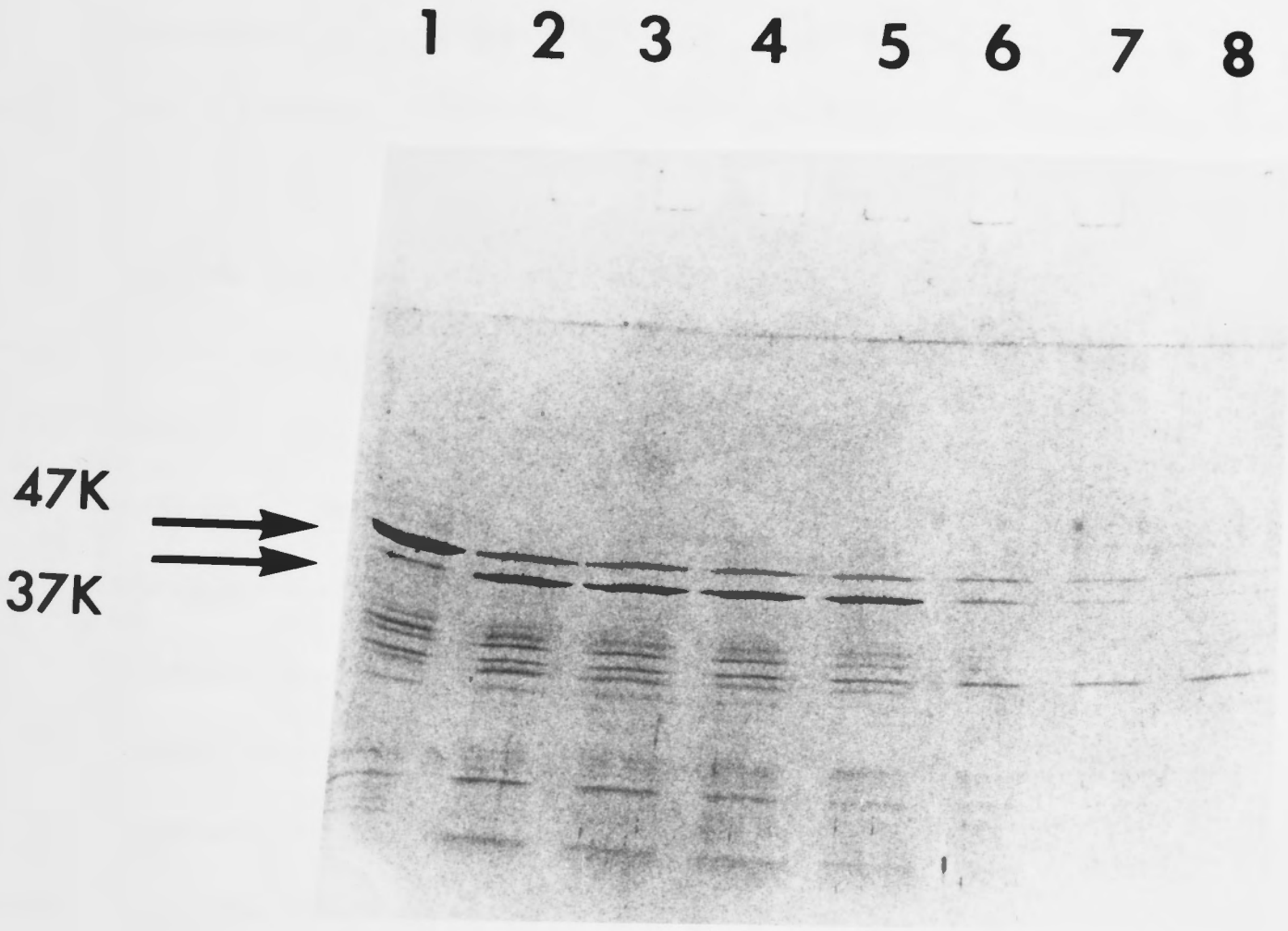
The purified enzyme was incubated in the presence of trypsin (2% w/w) at 0°C. Aliquots were withdrawn at the following times: (1) 0 hours, (2) 4 hours, (3) 8 hours, (4) 10 hours, and (5) 10 hours (without addition of trypsin) and derivatized in SDS at 100°C. See Materials and Methods for further details.

Figure 4-9. SDS-Polyacrylamide Gel Electrophoresis of NADH:ubiquinone Oxidoreductase after Proteolytic Digestion with Trypsin and Chymotrypsin



The purified enzyme was incubated in the presence of trypsin (2% w/w; samples 2-4) or chymotrypsin (2% w/w; samples 5-7) at 0°C. Aliquots were withdrawn at the following times: (1) 0 hours, (2) 2.5 hours, (3) 6 hours, (4) 7.5 hours, (5) 2.5 hours, (6) 6 hours (7) 7.5 hours and derivatized in SDS at 100°C. See Materials and Methods for further details.

Figure 4-10. SDS-Polyacrylamide Gel Electrophoresis of NADH:ubiquinone Oxidoreductase after Proteolytic Digestion with Pronase



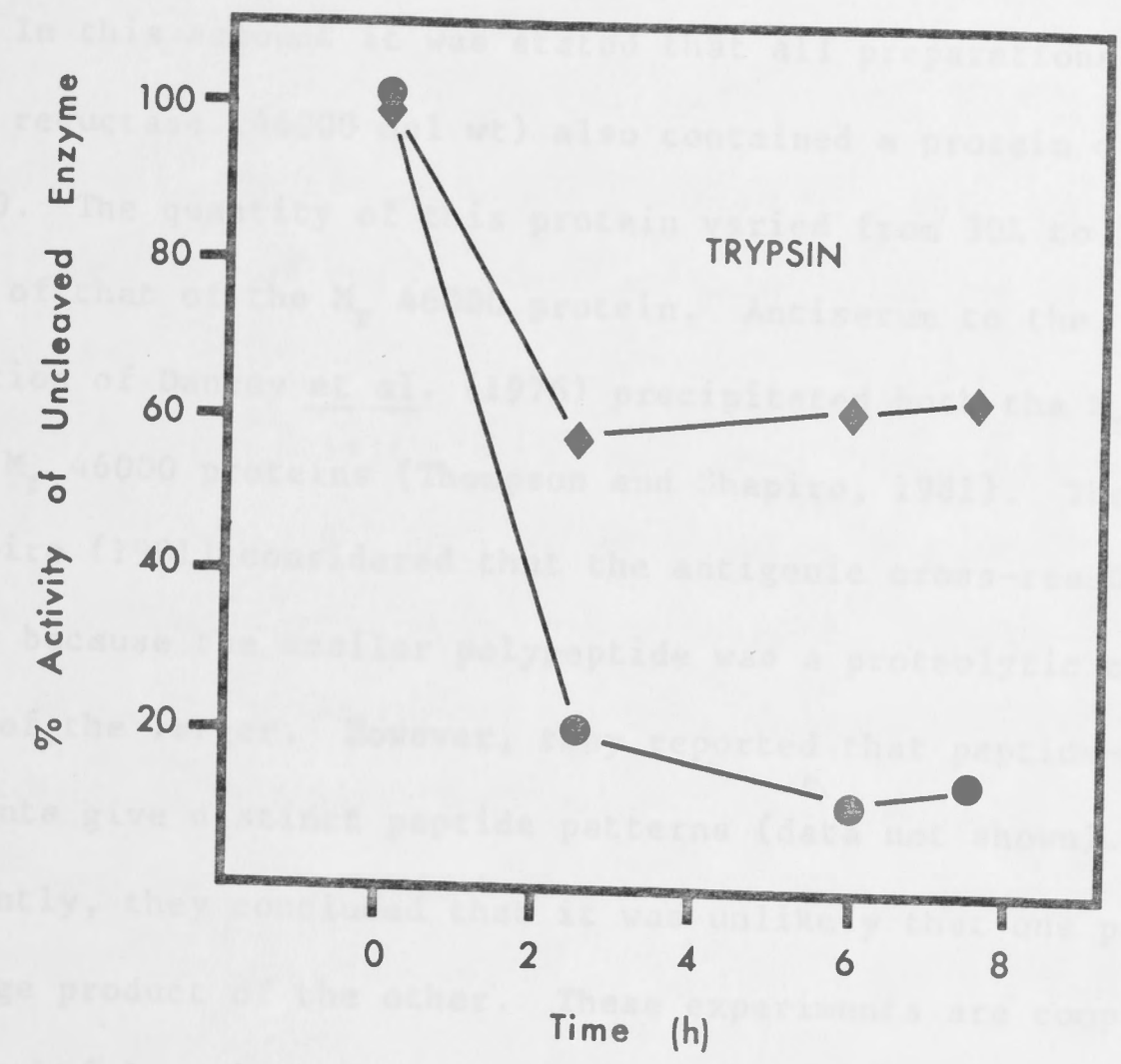
The purified enzyme was incubated in the presence of pronase (2% w/w) at 25°C. Aliquots were withdrawn at the following times: (1) 0 min, (2) 15 min, (3) 45 min, (4) 60 min, (5) 90 min, (6) 180 min, and (7) 225 min and derivatized in SDS at 100°C. See Materials and Methods for further details.

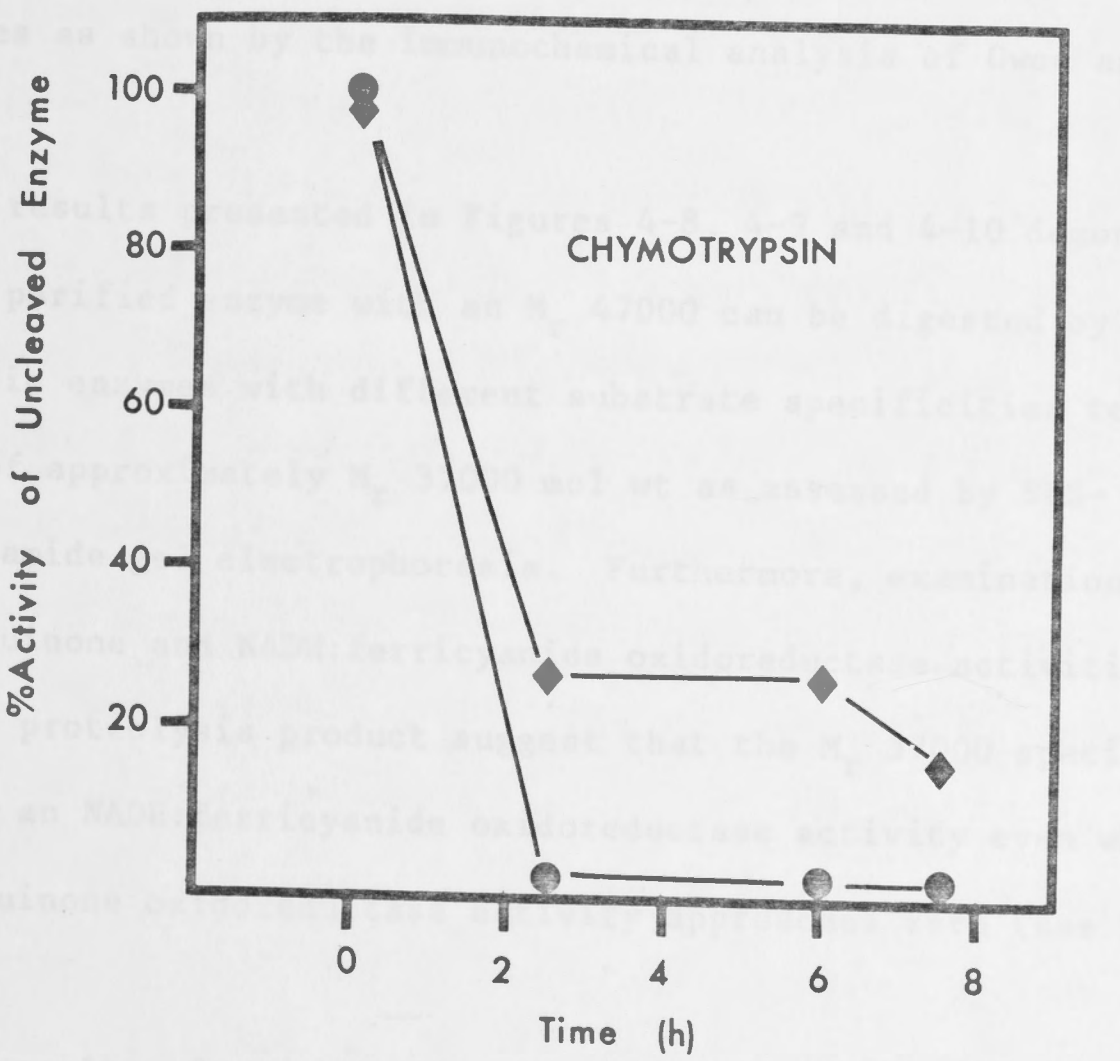
derivatization. Each was assayed for NADH:ubiquinone and NADH: $Fe(CN)_6^{3-}$ oxidoreductase activity. The recorded activities are illustrated as a percentage of undigested purified enzyme in Figure 4-11.

The ratio of activities towards ubiquinone-1 and $Fe(CN)_6^{3-}$ as acceptors are similar (4:1 respectively) for purified enzyme, wild type IY13 membrane vesicles and plasmid amplified IY91 membrane vesicles (Jaworowski et al., 1981a). It is clear from the results presented in Figure 4-11 that the ubiquinone-1 reductase and $Fe(CN)_6^{3-}$ reductase activities are reduced upon proteolysis. However, the $Fe(CN)_6^{3-}$ reductase activity is retained to a greater relative extent than the ubiquinone-1 reductase activity. This result is consistent with the hypothesis that the M_r 47000 species possesses both ubiquinone-1 and $Fe(CN)_6^{3-}$ reductase activities whereas the major proteolysis product(s) (the major species has an M_r of 37000 on a denaturing SDS-polyacrylamide gel) can transfer electrons to $Fe(CN)_6^{3-}$ but not to ubiquinone-1.

This interpretation could explain the original findings of Shapiro and coworkers (Dancey et al., 1976; Dancey and Shapiro, 1976; Thompson and Shapiro, 1981; see Chapter 1). Dancey et al. (1976) and Dancey and Shapiro (1976) reported that their purified preparations of the respiratory NADH dehydrogenase from E. coli had a mol wt of 38000 and possessed NADH:DCIP and NADH:$Fe(CN)_6^{3-}$ oxidoreductase activity. Although the artificial electron acceptors DCIP and ferricyanide were active as acceptors with the preparation, no ubiquinone reductase activity was quoted. In a later publication the respiratory NADH dehydrogenase was once again purified but the reported molecular weight had increased to M_r 46000 and

Figure 4-11. NADH:ubiquinone-1 and NADH:$Fe(CN)_6^{3-}$ Oxidoreductase Activity of Purified Enzyme after Proteolytic Digestion with Trypsin or Chymotrypsin





The purified enzyme was incubated in the presence of trypsin (2% w/w) or chymotrypsin (2% w/w) as described in Figure 4-9. Aliquots were removed and assayed for (●) NADH:ubiquinone and (◆) NADH:$Fe(CN)_6^{3-}$ oxidoreductase activity. The specific activity for the uncleaved enzyme was 230 and 120 units mg^{-1}, respectively. Assays were performed as described in the Materials and Methods (Chapter 3).

ubiquinone-3 reductase activity was cited (Thompson and Shapiro, 1981). In this account it was stated that all preparations of the quinone reductase (46000 mol wt) also contained a protein of M_r 37000. The quantity of this protein varied from 30% to less than 5% of that of the M_r 46000 protein. Antiserum to the preparation of Dancey et al. (1976) precipitated both the M_r 37000 and the M_r 46000 proteins (Thompson and Shapiro, 1981). Thompson and Shapiro (1981) considered that the antigenic cross-reactivity could be because the smaller polypeptide was a proteolytic cleavage product of the larger. However, they reported that peptide-mapping experiments give distinct peptide patterns (data not shown). Consequently, they concluded that it was unlikely that one protein is a cleavage product of the other. These experiments are complicated by the doubtful purity of the original preparation of the 38000 mol wt species as shown by the immunochemical analysis of Owen and Kaback (1979a).

The results presented in Figures 4-8, 4-9 and 4-10 demonstrate that the purified enzyme with an M_r 47000 can be digested by proteolytic enzymes with different substrate specificities to yield a product of approximately M_r 37000 mol wt as assessed by SDS-polyacrylamide gel electrophoresis. Furthermore, examination of the NADH:ubiquinone and NADH:ferricyanide oxidoreductase activities of the major proteolysis product suggest that the M_r 37000 species possesses an NADH:ferricyanide oxidoreductase activity even when the NADH:ubiquinone oxidoreductase activity approaches zero (see Figure 4-11).

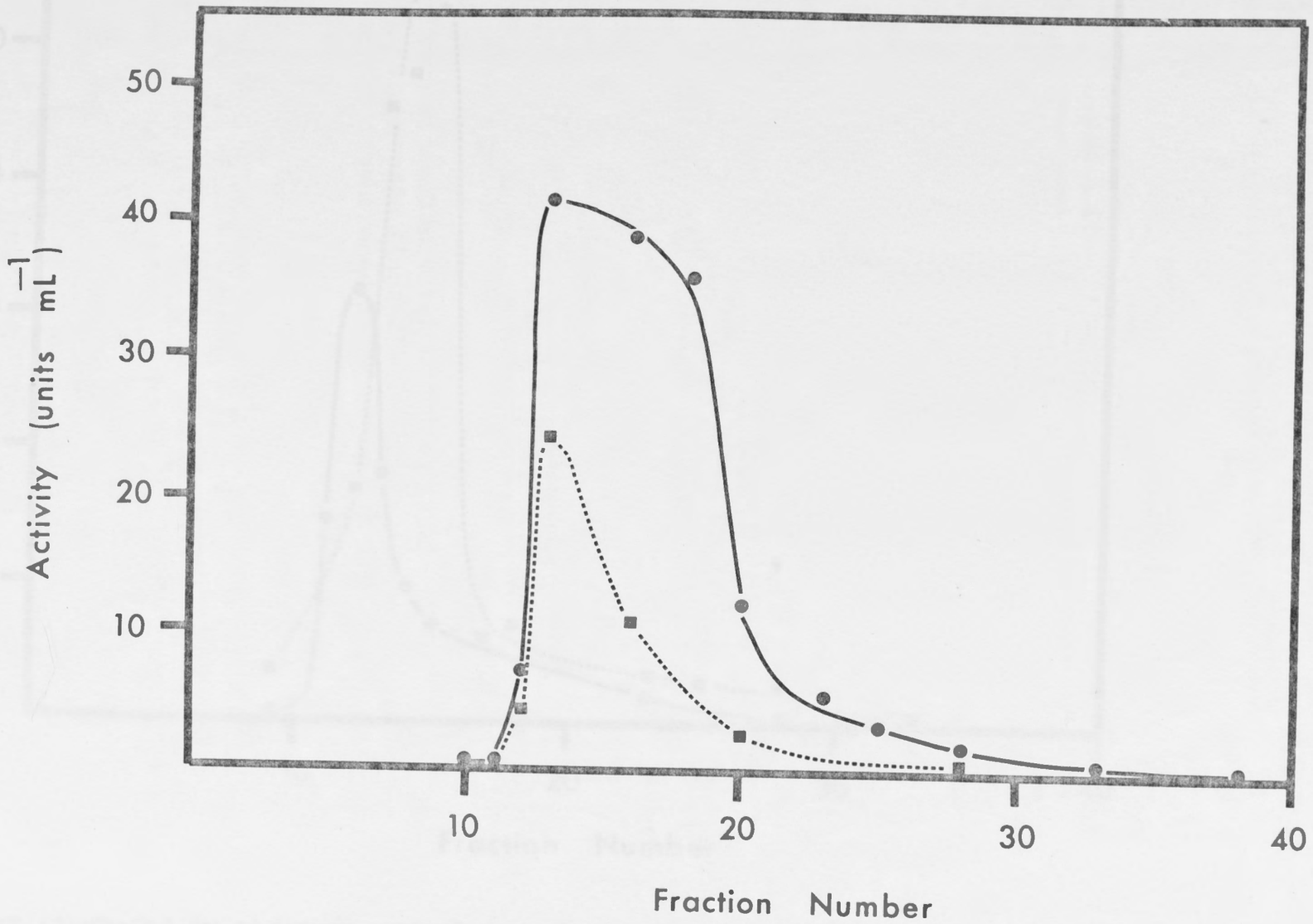
The results of the partial proteolysis experiments taken together with the results of Shapiro and colleagues suggested that it

is possible to generate a 37000 mol wt subfragment of the NADH dehydrogenase which possesses the NADH and flavin binding domains but has lost ubiquinone reductase activity. If such a subfragment arises from a single cleavage it might also be possible to isolate the small fragment of the enzyme which is required for the reduction of ubiquinone.

To investigate this further, concentrated purified enzyme was digested with trypsin and then chromatographed on a Sephadex G-75 column under conditions designed to maintain activity (see Materials and Methods). The chromatograms of undigested and trypsin digested enzyme are shown in Figures 4-12 and 4-13, respectively. Approximately 70% of the NADH:ubiquinone and NADH:$Fe(CN)_6^{3-}$ oxidoreductase activity was recovered in the undigested enzyme peak. However, with the digested enzyme the recovered NADH:ubiquinone oxidoreductase peak was only 4% whereas the NADH:$Fe(CN)_6^{3-}$ oxidoreductase activity was approximately 25%. The relatively higher recovery of the NADH:$Fe(CN)_6^{3-}$ oxidoreductase activity is interesting when it is considered that the peak has been shifted to a position of lower mol wt as illustrated in Figure 4-13. The reason why the NADH:ubiquinone oxidoreductase peak is so broad in Figure 4-12 is unknown. A sample of each enzyme preparation was taken prior to Sephadex G-75 chromatography and electrophoresed on an SDS-polyacrylamide gel (Figure 4-14). The predominant band in the digested enzyme preparation was the M_r 37000 species.

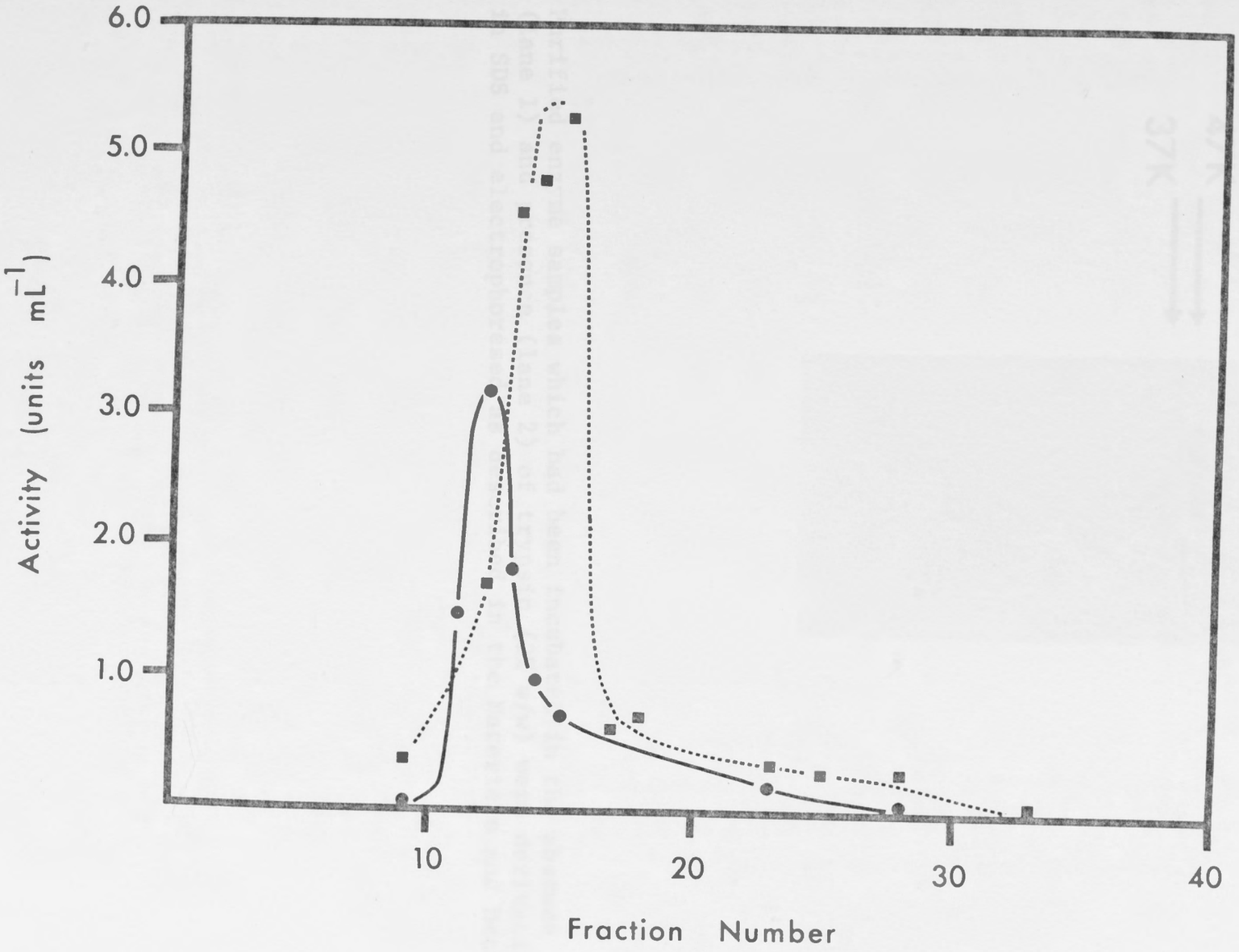
These results suggested that the NADH dehydrogenase might have a similar organisation to cytochrome $\underline{b}_5$ and cytochrome $\underline{b}_5$ reductase. According to this model the small (~8000) 'clipped off' portion would be at the C-terminus and would constitute a putative membrane-

Figure 4-12. Sephadex G-75 Chromatogram of Purified NADH:ubiquinone Oxidoreductase



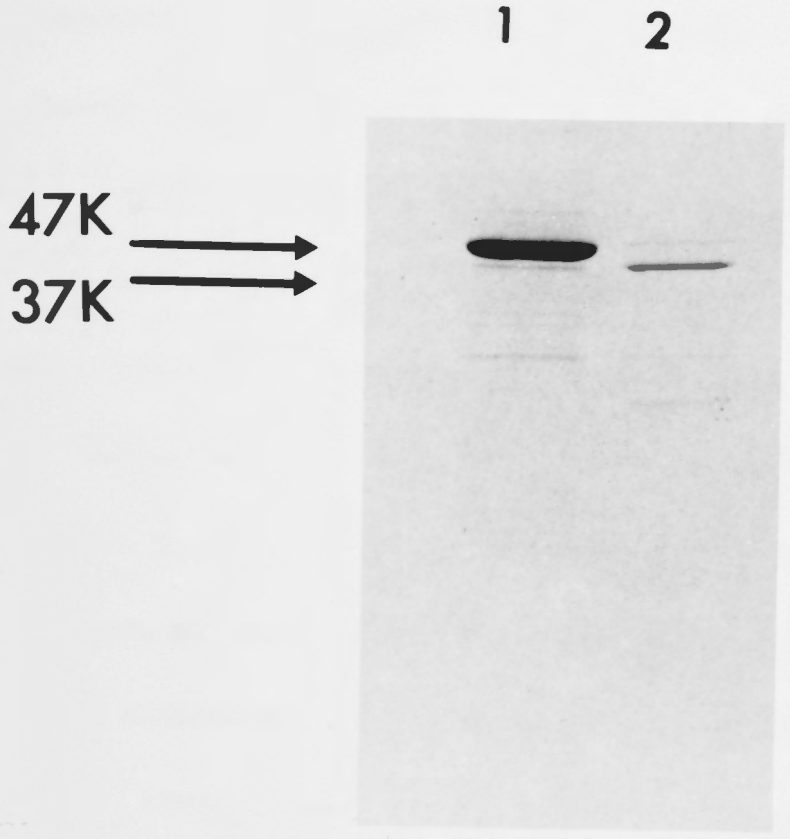
Purified enzyme was chromatographed under conditions described in the Materials and Methods section. Each fraction was assayed for (●) NADH:ubiquinone and (■) NADH:$Fe(CN)_6^{3-}$ oxidoreductase activity as described in Chapter 3.

Figure 4-13. Sephadex G-75 Chromatography of NADH:ubiquinone Oxidoreductase after Proteolytic Digestion with Trypsin



Purified enzyme which had been incubated in the presence of trypsin was chromatographed under conditions described in the Materials and Methods section. Each fraction was assayed for (●) NADH:ubiquinone and (■) NADH:$Fe(CN)_6^{3-}$ oxidoreductase activity as described in Chapter 3.

Figure 4-14. SDS-Polyacrylamide Gel Electrophoresis of NADH:ubiquinone Oxidoreductase (Digested with Trypsin) prior to Sephadex G-75 Chromatography



Purified enzyme samples which had been incubated in the absence (lane 1) and presence (lane 2) of trypsin (2% w/w) were derivatized in SDS and electrophoresed as described in the Materials and Methods.

associated tail. This portion of the protein would be required for the interaction with ubiquinone (see Chapter 3).

In support of the hypothesis outlined above two aspects of the primary amino acid sequence can be cited. Firstly, in the region of amino acid residues 352-362 (see Chapter 2) there are six potential proteolytic cleavage sites; three lysines (trypsin) and two tyrosines and one histidine (chymotrypsin). A single or even multiple cleavages at any of these sites would produce two peptides of M_r ~38000 and M_r ~9400, respectively. There are no other concentrations of possible cleavage sites either from the N- or C-terminal end which would produce the observed M_r 37000 band. Secondly, the N-terminus carries part of a nucleotide binding domain (see Chapter 2). Hence, if the N-terminus was cleaved upon proteolysis it is unlikely that the product would be able to transfer electrons to ubiquinone-1 or $Fe(CN)_6^{3-}$ at appreciable rates. Thirdly, uncharged regions of the amino acid sequence would be the most likely candidates for membrane association. The largest uncharged stretches are near the C-terminal end of the protein at residues 331-351 (21 residues), 362-382 (21 residues), 402-412 (10 residues) and 414-424 (10 residues) (see Chapter 2).

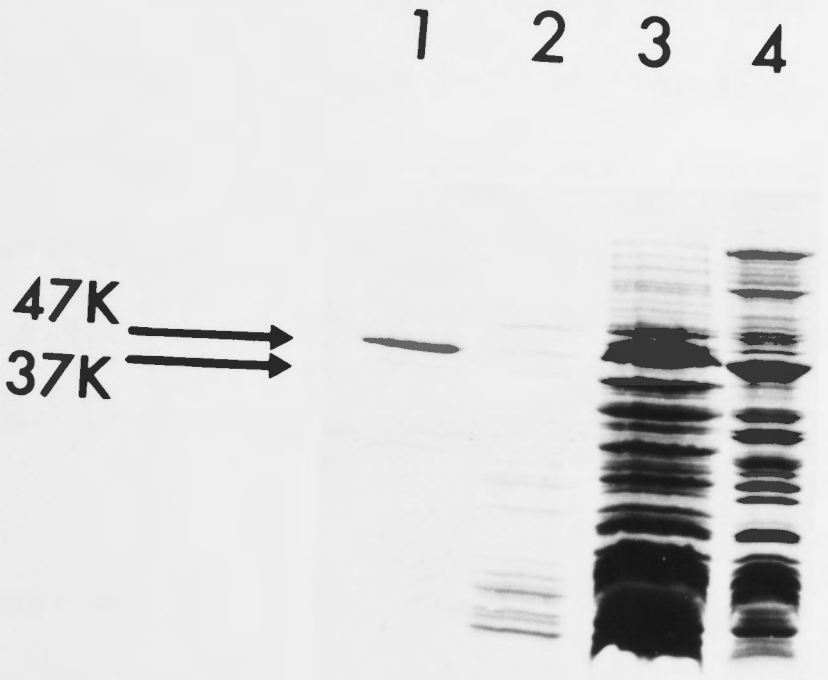
In an attempt to examine the possibility that the C-terminal section of the enzyme is both membrane associated and the site of ubiquinone interaction, proteolysis studies of membrane vesicles were initiated.

IY91 membrane vesicles were digested by chymotrypsin, the membranes pelleted by ultracentrifugation and then resuspended to their original volume. Aliquots were removed at each stage of the procedure for assay and analytical SDS-polyacrylamide gel

electrophoresis (Figure 4-15). One of the important features of the electrophoretogram is that upon chymotrypsin digestion of IY91 membrane vesicles a major peptide is not released into the supernatant under the conditions used. The M_r 37000 dominant cleavage product is clearly still membrane-associated (see lane 3, Figure 4-15). The percentage of the undigested M_r 47000 species was calculated (adjusted for the volume of membranes loaded). Only 23% of the original M_r 47000 species was intact and 18% was present as the M_r 37000 species (assuming equal staining with dye). The fate of the other 60% of the M_r 47000 band is unknown but presumably upon cleavage some of the enzyme is further cleaved to give multiple cleavage products which are not easily identified.

The protein recoveries calculated from the SDS-polyacrylamide gel are not reflected in the amount of NADH:ubiquinone and NADH:$Fe(CN)_6^{3-}$ oxidoreductase activities recovered in the digested membrane preparation. The chymotrypsin digested membrane preparation when assayed still maintained 65% and 61% respectively, of its original activities. Therefore, it must be concluded that although the M_r 47000 enzyme is digested >75%, the enzymic activity is largely maintained suggesting that in the membrane environment the cleaved enzyme is stabilized until disrupted by SDS derivatization. This result is in contrast to those obtained with purified enzyme (Figures 4-9 and 4-11), where the extent of proteolysis is proportional to the loss of enzymic activity. Possibly the membrane environment helps to maintain the overall enzyme configuration. Interestingly, Michell and Weitzman (1983) demonstrated that subtilisin digested Acinetobacter citrate synthase maintained its co-operative interactions characteristic of the native enzyme even

Figure 4-15. SDS-Polyacrylamide Gel Electrophoresis of IY91 Membrane Vesicles after Proteolytic Digestion with Chymotrypsin



IY91 membrane vesicles were digested with chymotrypsin (2% w/w, 0°C), the membranes pelleted by ultracentrifugation and then resuspended to their original volume (1.0 mL in STM buffer). Aliquots were removed at each stage of the procedure, SDS derivatized, and electrophoresed as described in the Materials and Methods. Samples include: (1) purified enzyme, (2) supernatant of pelleted chymotrypsin digested IY91 membranes, (3) resuspended pellet of chymotrypsin digested IY91 membranes, and (4) untreated IY91 membranes.

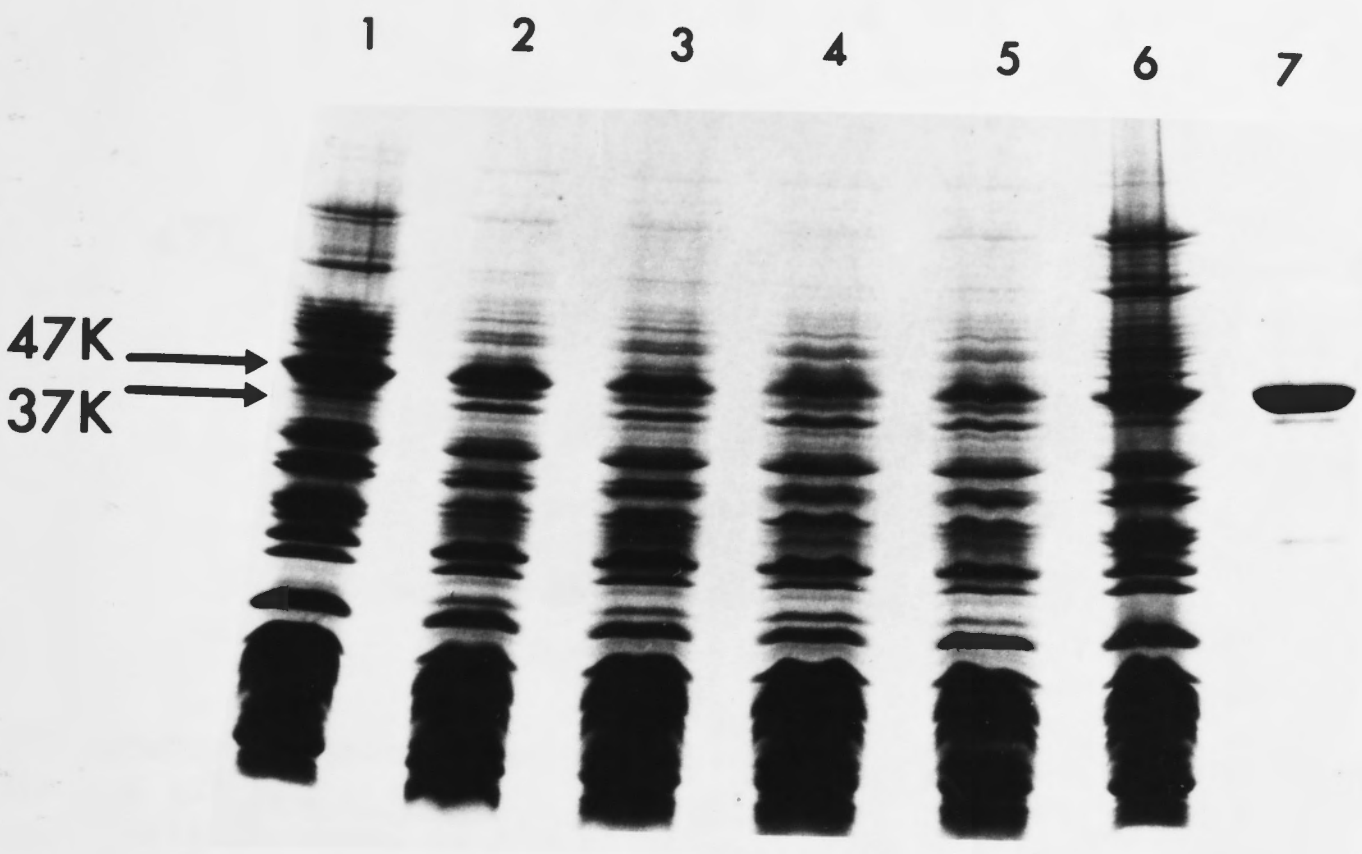
though SDS-polyacrylamide gel electrophoresis clearly revealed breakdown of the digested enzyme into smaller fragments.

Digestion of IY91 membrane vesicles by trypsin also yielded the M_r 37000 proteolytic product (Figure 4-16). Both the NADH:ubiquinone and NADH:$Fe(CN)_6^{3-}$ oxidoreductase activities are >85% of the untreated IY91 membrane vesicle levels even though they are >50% degraded.

The broad-specificity proteases such as pronase and subtilisin make short work of the enzyme leading to complete digestion and loss of activity of the M_r 47000 and M_r 37000 species (see Figures 4-17 and 4-18). Possibly a significant amount of the enzyme is exposed (ie little is embedded in the membrane) and once digestion is initiated then proteolysis rapidly leads to the destruction of the enzyme. Why this should occur with the enzyme in the membrane (Figure 4-17) and not with the purified enzyme is puzzling (Figure 4-10). The proteolysis experiments involving the purified enzyme (Figures 4-8, 4-9 and 4-10) were performed in low ionic strength buffer. The fact that the purified enzyme is in an aggregated state in this buffer (Figure 4-2) may play some part in protecting the enzyme from rapid and complete digestion with pronase (Figure 4-10).

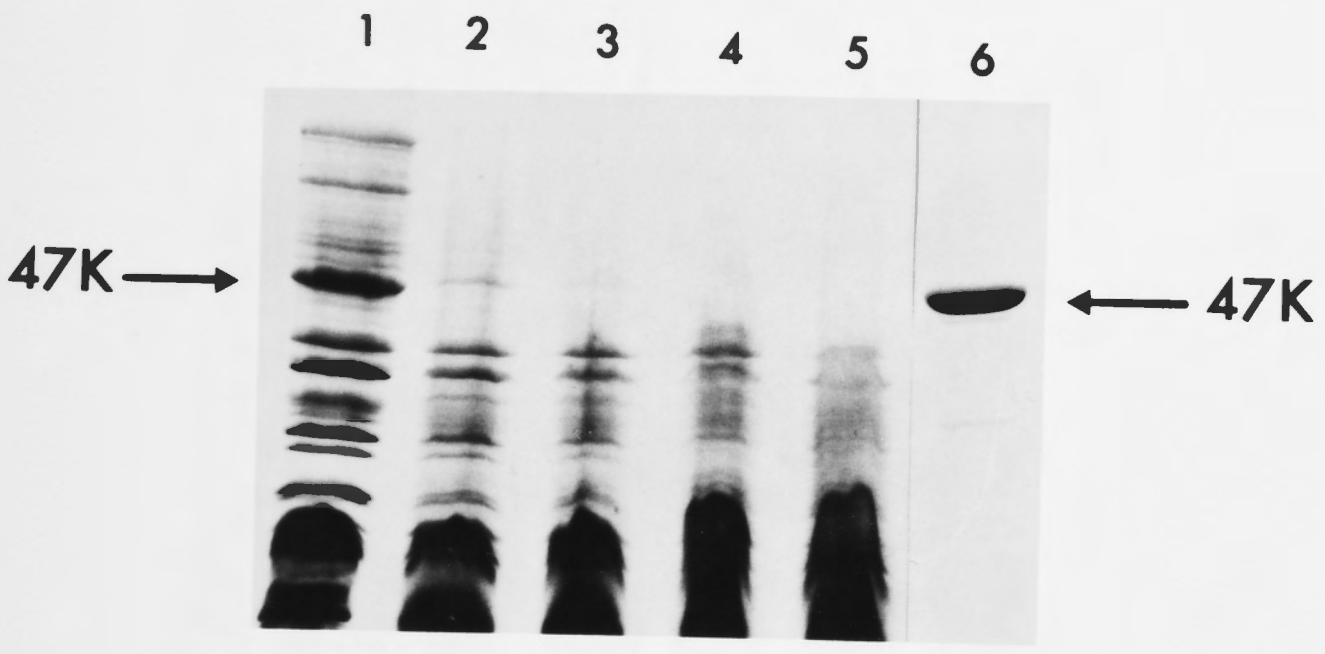
The results with the proteolysis of IY91 membranes by pronase and subtilisin suggest that a significant amount of the enzyme is exposed. This is not surprising when it is considered that a large proportion of the enzyme shows some homology with the soluble enzyme glutathione reductase (see Chapter 2). Furthermore, the enzyme must interact in the cytoplasm with the soluble substrate NADH.

Figure 4-16. SDS-Polyacrylamide Gel Electrophoresis of IY91 Membranes after Proteolytic Digestion with Trypsin



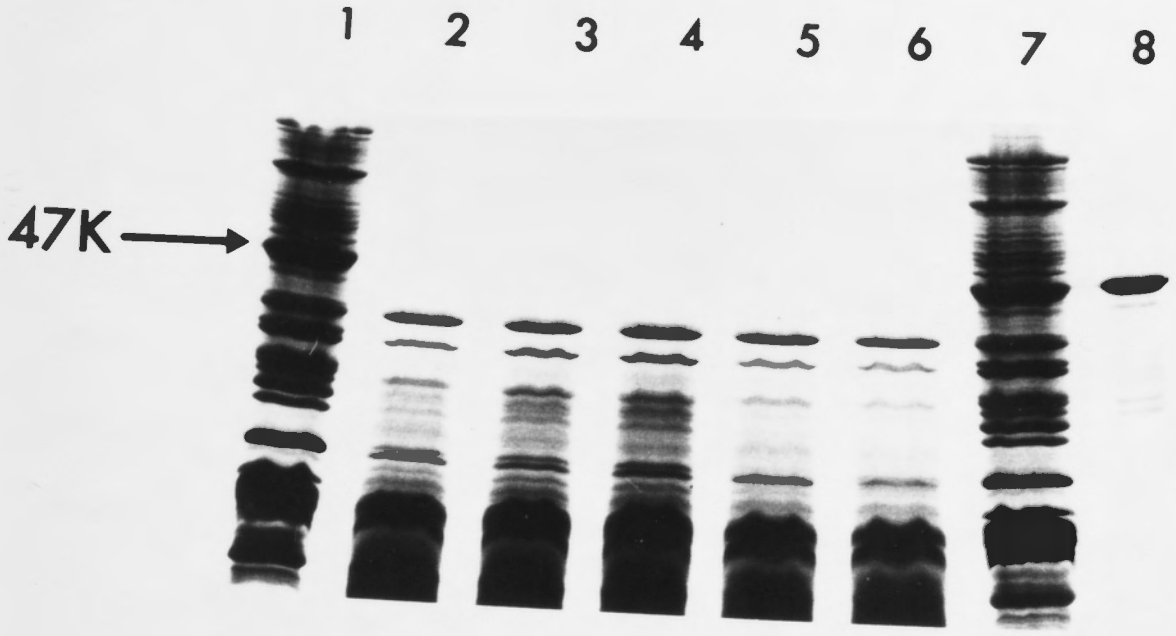
IY91 membrane vesicles were incubated at 25°C in the presence of trypsin (2% w/w). Aliquots were withdrawn and derivatized in SDS and electrophoresed as described in the Materials and Methods. Samples include: (1) 0 min, (2) 30 min, (3) 60 min, (4) 180 min, (5) 240 min, (6) 240 min (no addition of trypsin), and (7) pure enzyme (no addition of trypsin).

Figure 4-17. SDS-Polyacrylamide Gel Electrophoresis of IY91 Membranes after Digestion with Pronase



IY91 membrane vesicles were incubated at 25°C in the presence of pronase (2% w/w). Aliquots were withdrawn and derivatized in SDS and electrophoresed as described in the Materials and Methods. Samples include: (1) 0 min (no pronase added), (2) 30 min, (3) 60 min, (4) 180 min, (5) 240 min, and (6) pure enzyme (no pronase added).

Figure 4-18. SDS-Polyacrylamide Gel Electrophoresis of IY91 Membranes after Proteolytic Digestion with Subtilisin



IY91 membrane vesicles were incubated at 25°C in the presence of subtilisin (2% w/w). Aliquots were withdrawn and derivatized in SDS and electrophoresed as described in the Materials and Methods. Samples include: (1) 0 min (no subtilisin added), (2) 10 min, (3) 20 min, (4) 30 min, (5) 60 min, (6) 180 min, (7) 180 min (no subtilisin added), and (8) pure enzyme (no subtilisin added).

The proteolysis results with the purified enzyme are consistent with a possible ubiquinone binding domain at the C-terminus. Also consistent with this suggestion is the demonstration that the purified enzyme can reduce ubiquinone-8 (see Chapter 3) and that the C-terminus is the most hydrophobic region of the protein (see Chapter 2). However, the results obtained concerning the digestion of the IY91 membrane vesicles do not suggest that the enzyme is attached to the membrane by a small anchor which is susceptible to proteolysis such as is the case with cytochrome $\underline{b}_5$ and cytochrome $\underline{b}_5$ reductase.

SUMMARY

The reconstitution of membrane vesicles of *ndh* mutants by purified NADH dehydrogenase has been investigated. The enzyme can readily become membrane-associated and reconstitute the NADH oxidase of membrane vesicles from *ndh* mutants. Also, the purified enzyme has been shown to be capable of inserting into the outer surface of the inner cytoplasmic membrane of *ndh* spheroplasts. Once assembled, the enzyme does not readily 'flip' across the lipid bilayer even when amplified to 70-fold wild type levels. The observation that the purified enzyme can reconstitute *ndh* spheroplasts suggests that there is no obligatory protein receptor that facilitates membrane binding.

It has been shown by sucrose density centrifugation that in 0.7 M potassium phosphate buffer the purified enzyme migrates with an M_r of 80000 and an $S_{20,w}$ of 6.3. In contrast to these figures, the purified enzyme in low ionic strength potassium phosphate buffer was shown to be in a highly aggregated form. The pooled activity peaks

from these gradients when added to ndh membrane vesicles demonstrated that the disaggregated enzyme preparation was significantly more reconstitutively active than the aggregated preparation.

Proteolysis of the purified NADH:ubiquinone oxidoreductase and IY91 membranes has led to the partial characterization of a major proteolytic degradation product with an M_r of ~37000. Its NADH:ubiquinone and NADH:$Fe(CN)_6^{3-}$ oxidoreductase relative activities as well as its M_r suggest that it is the same species isolated by Dancey and Shapiro (1976) and Dancey et al., (1976). Proteolysis of the purified enzyme with either trypsin or chymotrypsin leads to a significant loss of NADH:ubiquinone oxidoreductase activity. Interestingly, there is relatively less of a reduction in the NADH:$Fe(CN)_6^{3-}$ oxidoreductase activity. Evidence is provided that is consistent with the suggestion that proteolytic cleavage occurs at the C-terminal region which is postulated to play a significant role in ubiquinone reduction.

The M_r 37000 species is still membrane-associated after proteolysis of IY91 membranes with trypsin and chymotrypsin and maintains its functional activity. This suggests that its interaction with the membrane or with its membrane embedded segment(s) is sufficient to allow it to bind and pellet with the membrane in a functional conformation. Proteolysis of IY91 membranes with pronase and subtilisin demonstrated that a significant amount of the enzyme is exposed. Furthermore, the proteolysis experiments carried out with IY91 membranes and trypsin and chymotrypsin suggest that the enzyme is not anchored in the membrane by a short polypeptide that is susceptible to proteolytic cleavage.

Chapter 5

Novel Photoaffinity Analogues of Ubiquinone: Radiochemical Labelling of the Respiratory NADH Dehydrogenase

INTRODUCTION

Affinity Labelling

An understanding of the mechanism of action of enzyme-catalyzed reactions requires the identification of the amino acids in the active site involved in binding and those more directly involved in catalysis. The classical approach to acquiring such information has been the use of general and specific reagents for chemical modification and ultimately identification of active site residues, commonly referred to as affinity labelling.*

Another approach to chemical modification of active site residues has been successfully applied, that is suicide inhibition (for review, Rando, 1977). This method makes use of the catalytic function of enzymes to generate a reactive molecule in the active site from an inert precursor. The reactive molecule then reacts with nucleophilic residues of the enzyme. As pointed out by Chowdhry and Westheimer (1979), affinity labelling in general, and suicide inhibition in particular, require the presence of appropriately positioned reactive functional groups on the enzyme to bring about inactivation and labelling.

Ideal labelling reagents should react not only with nucleophiles but even with the significantly less reactive but ubiquitous hydrophobic regions (C-H bonds) of proteins. Photogenerated reagents have the potential for high reactivity and also can be activated in situ. Photoaffinity labelling has become a major technique for studying molecular interactions in biological systems (for comprehensive reviews see Bayley and Knowles, 1977;

*The field of affinity labelling has become such a powerful tool that a whole volume of Methods in Enzymology has been devoted to it (Vol XLVI, 1977, W.B. Jacobs and M. Wilchek, Academic Press).

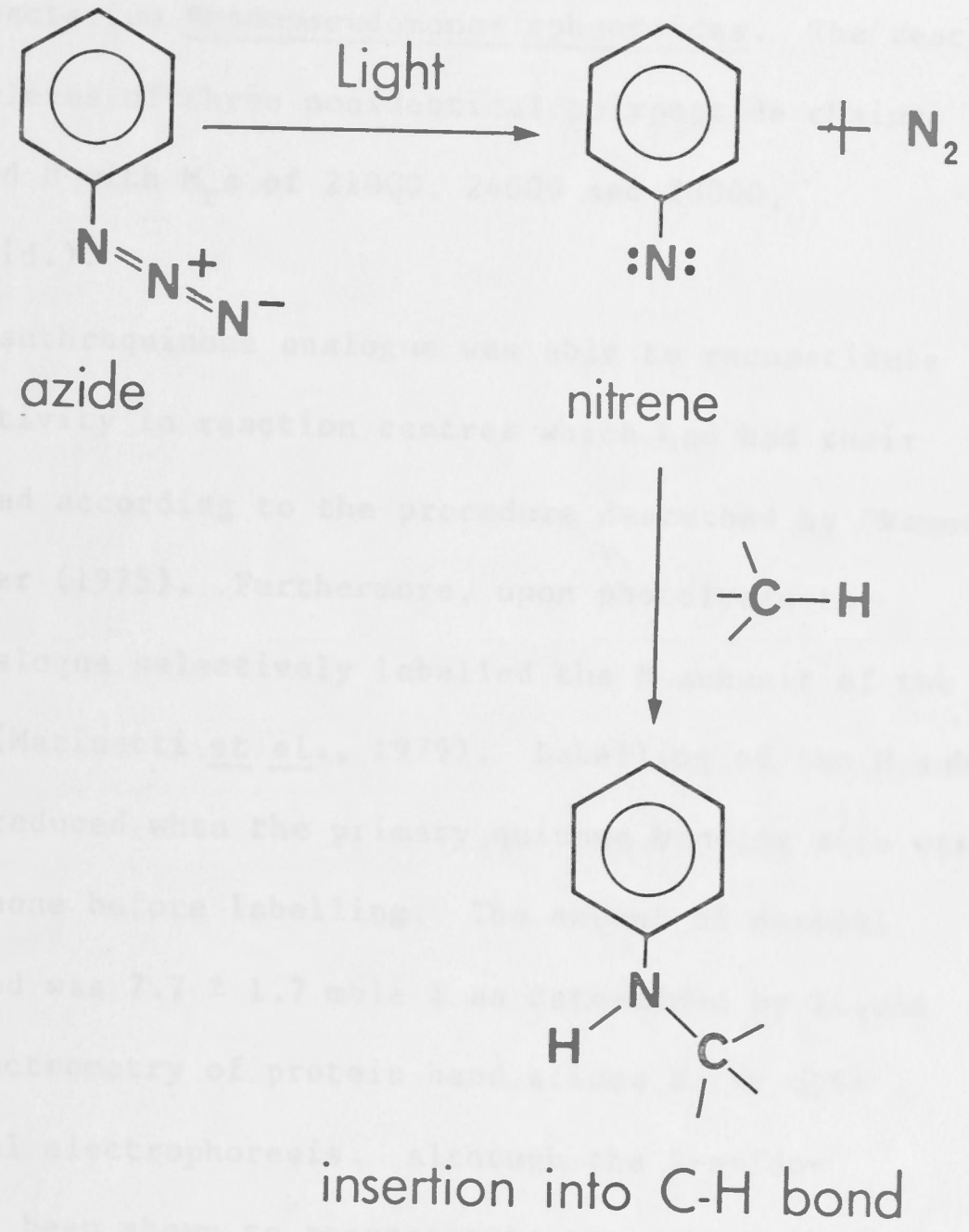
Chowdhry and Westheimer, 1979). A photolabile reagent is anchored to the macromolecule, when possible by a covalent bond, photolysis of the complex then leads to the generation of a highly reactive species that labels the macromolecule or specifically the active site (see Figure 5-1). Ideally, a very reactive reagent is generated only at the ligand binding site of interest, and reacts indiscriminately with whatever chemical groups it finds there.

The properties desirable in a photolabile group are chemical stability prior to photoactivation, rapid rate of photolysis at long wavelength, and a very short half life for the photogenerated reactive species. The mechanism of labelling should not involve intramolecular rearrangement to a less reactive labelling species.

Carbenes and nitrenes are the principal reagents utilized for photoaffinity studies in biological systems (Bayley and Knowles, 1977). The arylnitrene generating reagents have been suggested as being the most ideal photoaffinity analogues for use in the active sites of enzymes (Guillory, 1979).

With the aim of constructing pyridine nucleotide photoaffinity analogues, Jeng and Guillory (1975) synthesized what has become a useful photoaffinity group, N-4-azido-2-nitrophenyl-β-alanine (ANAP), which can be readily linked via esterification to alcohols. Guillory and Jeng (1977) later described methods which would enable the synthesis of [^{3}H]- and [^{14}C]-labelled arylazido-nucleotide analogues. Subsequently, the [^{3}H]-arylazido-β-alanyl NAD^{+} has been used to radiochemically label the M_r 57000 subunit of purified mitochondrial NADH dehydrogenase (Chen and Guillory, 1981) and to indicate that complex I has three distinct nucleotide reactive sites (Chen and Guillory, 1979; 1980).

Figure 5-1. Schematic Representation of the Photolysis of an Azide and Insertion of the Generated Nitrene into a C-H Bond



Quinone Photoaffinity Analogues

Marinetti, Okamura and Feher (1979) synthesized a [^{3}H]-labelled 2-azidoanthraquinone derivative to probe reaction centres of the photosynthetic bacterium Rhodopseudomonas sphaeroides. The reaction centres are complexes of three nonidentical polypeptide chains denoted L, M, and H with M_rs of 21000, 24000 and 28000, respectively (ibid.).

The 2-azidoanthraquinone analogue was able to reconstitute photochemical activity in reaction centres which had had their ubiquinone removed according to the procedure described by Okamura, Isaacson and Feher (1975). Furthermore, upon photolysis the photoaffinity analogue selectively labelled the M subunit of the reaction centre (Marinetti et al., 1979). Labelling of the M subunit was shown to be reduced when the primary quinone binding site was filled by ubiquinone before labelling. The extent of maximal labelling achieved was 7.7 ± 1.7 mole % as determined by liquid scintillation spectrometry of protein band slices after SDS-polyacrylamide gel electrophoresis. Although the 2-azido-anthraquinone had been shown to reconstitute photochemical activity in ubiquinone depleted reaction centres (Marinetti et al., 1979), it is noteworthy that anthraquinone had previously been shown to deviate from ubiquinone in low-temperature decay kinetics in the reaction centres (Okamura et al., 1975). This may be due to the fact that anthraquinone is structurally very different from ubiquinone in that it has three six-membered rings rather than the benzoquinone structure.

Yu and Yu (1980b; 1982) synthesized a functionally active arylazido-1-[^{14}C]-β-alanine-ubiquinone derivative (Q_0C_{10}NAPA)

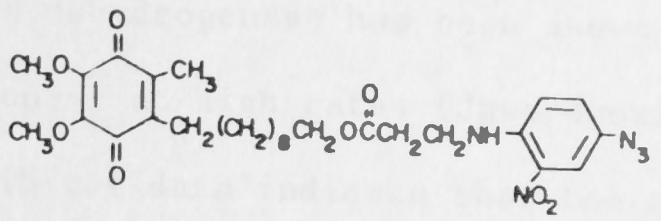
in an attempt to identify the ubiquinone binding protein in ubiquinol-cytochrome c reductase preparations (see Figure 5-2 for Q_0C_{10}NAPA structure).

The photoaffinity analogue, Q_0C_{10}NAPA, was prepared by the esterification of $Q_0(CH_2)_{10}OH$ and arylazido-β-alanine (Yu and Yu, 1982). The derivative has one notable deficiency. The photoactivatable azido group is very distant from the quinone ring. Yu and Yu (1980b) reported that the arylazido-1-[^{14}C]-β-alanine ubiquinone derivative labelled some specific proteins after photolysis although the counts incorporated were very low and no stoichiometry of label/protein was cited.

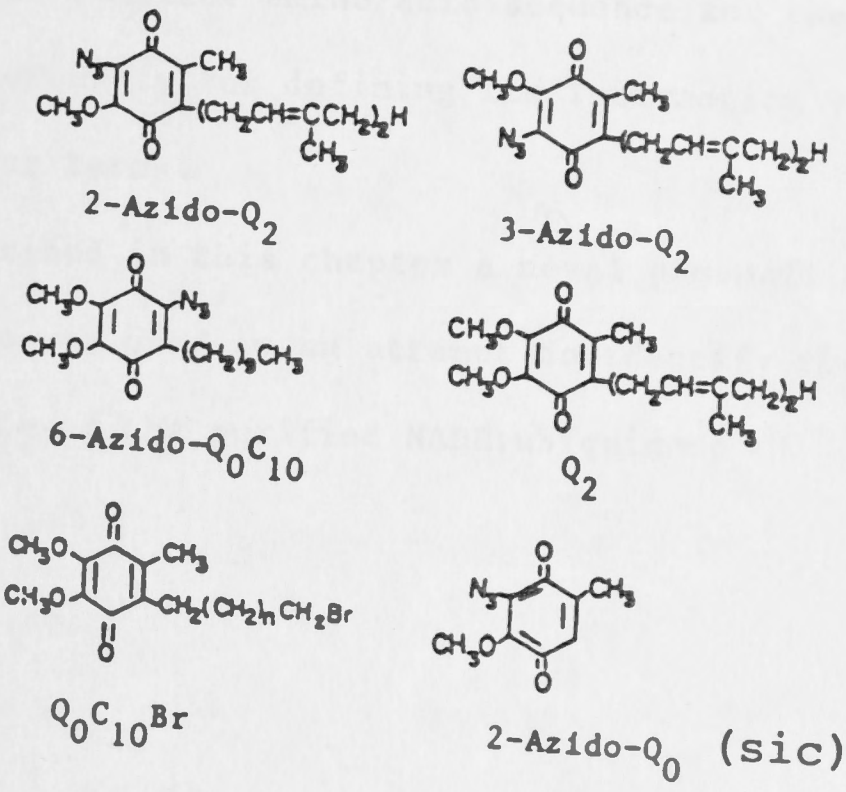
Yu, Gu and Yu (1982) synthesized a further series of azidoubiquinone analogues which possessed the azide reporter group on the 2, 3 or 6 position on the quinone ring (Figure 5-2). Yu et al. (1982) reported that the analogues were able to act as electron acceptors and photolysis in their presence led to the inhibition of the succinate cytochrome c reductase system. Unfortunately, the extent of labelling by the new reagents was not given.

The synthesis of azidoubiquinone analogues possessing photoactivatable groups on the quinone ring is a significant advance over the previous Q_0C_{10}NAPA analogue. However, some authors have noted that analogues with the azide moiety on the quinone ring photodecompose by internal rearrangement without generating nitrenes (Marinetti et al., 1979; Germeraad, Weyler and Moore, 1974). The details of the synthetic procedures for the azidoubiquinone analogues reported by Yu et al. (1982) have not yet been published.

Figure 5-2. Azidoubiquinone Photoaffinity Analogue



Structure of $Q_0C_{10}NAPA$
(from Yu and Yu, 1980b)



Structure of azidoubiquinone derivatives (from Yu, Gu and Yu, 1982)

Photoaffinity Labelling of the Respiratory NADH Dehydrogenase

The respiratory NADH dehydrogenase has been shown to catalyze the reduction of ubiquinone-1 at high rates (Jaworowski *et al.*, 1981a). Kinetic and inhibitor data indicate that the enzyme possesses a ubiquinone binding site (see Chapter 3). The complete DNA sequence of the *ndh* gene and the deduced amino acid sequence of the enzyme has been determined (Young *et al.*, 1981; see Chapter 2). The availability of the complete amino acid sequence for the enzyme provides a unique opportunity for defining its interaction with ubiquinone in molecular terms.

In the work described in this chapter a novel photoaffinity analogue of ubiquinone was used in an attempt to identify the ubiquinone binding site of the purified NADH:ubiquinone oxidoreductase.

MATERIALS AND METHODS

Chemicals

Ubiquinone-1 was supplied by F. Hoffman-La Roche and Co., Basel and also by D. Magrath of this laboratory. The purity of the ubiquinone-1 was monitored by thin layer chromatography (TLC) and by ultraviolet absorption spectroscopy and mass spectrometry. Fluka supplied boron trifluoride diethyl etherate (laboratory reagent). [1-^{14}C]-3-aminopropanoic acid (54.7 Ci/mole) was from New England Nuclear. Sodium borohydride and silica gel TLC plates (F_{254}) were from Merck. Ethanol and methanol (Merck) were redistilled before use.

A number of chemicals were synthesized by Dr H.D. Campbell in this laboratory using published methods. These include: (a) BNPS-skatole (2-(2-nitrophenylsulphenyl)-3-methyl-3-bromoindolenine) (Omenn, Fontana and Anfinsen, 1970; Fontana, 1972), (b) ANAP (N-4-azido-2-nitrophenyl-β-alanine) (Jeng and Guillory, 1975; Guillory and Jeng, 1977), and (c) [^{14}C]-ANAP, which was synthesized by reacting [1-^{14}C]-3-amino propanoic acid (50-100 μCi) with FNPA (4-fluoro-3-nitrophenyl azide) as described previously (Guillory and Jeng, 1977). The optimal conditions for the cleavage reaction involving BNPS-skatole were determined by Dr H.D. Campbell and were based upon the procedures reported by Omenn et al. (1970) and Fontana (1972).

Spectrometry

Proton magnetic resonance spectra were recorded by Dr D. Fenn using a JEOL FX-90Q Fourier nuclear magnetic resonance spectrometer at 30°C. Samples (~1 mg) were dissolved in $CDCl_3$ (~0.5 mL) and chemical shifts are expressed relative to the internal standard tetramethylsilane (TMS). Typically, 100-500 pulses were accumulated.

Electron impact (70 eV) and chemical ionization (NH_3) mass spectra were recorded by Dr J.K. Macleod using a VG-micromass 7070F mass spectrometer fitted with a direct insertion probe.

Visible and ultraviolet absorption spectra were recorded on a Cary 118 spectrophotometer.

Liquid scintillation spectrometry was performed using a Packard Tri-carb 460 CD liquid scintillation spectrometer with a xylene-

Triton X-100 based scintillant (33% v/v Triton X-100; ~67% v/v xylene; 0.5% (w/v) 2,5-diphenyloxazole).

Reconstitution of Oxidase Activity

Membrane preparations of the E. coli strains AN384 (ubi men) and AN387 were prepared as described previously (see Chapter 3; Wallace and Young, 1977b). AN384 and AN387 are isogenic strains prepared by P1-transduction.

The oxidases present in respiratory membranes were estimated by measuring the rate of oxygen uptake using a Clark type oxygen electrode (Wallace and Young, 1977b). The reaction mixture contained 50 µL of membranes (~2 mg protein) and substrates (0.6 mM NADH, 20 mM succinate or 4 mM D-lactate) in a total volume of 2.5 mL STM buffer.

Amino Acid Analysis

Amino acid analyses were performed by Dr D.C. Shaw using a Beckman 120C amino acid analyzer.

Synthesis of 2'-Hydroxyubiquinone-1 and 2'-Arylazidoubiquinone-1 (2'-ANAP-Q-1)

2'-Hydroxyubiquinone-1 was prepared from ubiquinone-1. In order to synthesize the 2'-hydroxyubiquinone-1 the following synthetic procedure was used: 8 mg of $NaBH_4$ was added to 37 µmole ubiquinone-1 in 400 µL of dried tetrahydrofuran (THF). Hydroboration of the sidechain double bond was then accomplished using diborane generated in situ by the addition of 64 µL boron trifluoride etherate plus 236 µL THF. The mixture was then incubated at room temperature for 2 hours protected from light. The resulting alkylborane was oxidized

with alkaline hydrogen peroxide to yield the quinol form of 2'-hydroxyubiquinone-1. This was done by the sequential addition of 120 µL water (5 minutes incubation), 20 µL NaOH (30% w/v) and then 20 µL of H_2O_2 (30% w/v). The solution was then acidified with 75 µL of 2N H_2SO_4 and the quinol was extracted with 3 x 1 mL $CHCl_3$. The quinol was oxidized by the addition of Ag_2O (15 mg) to the $CHCl_3$ extract. The mixture was then shaken for 3 hours protected from light and filtered through celite. The celite was washed with $CHCl_3$ (~40 mL). The combined filtrates were rotary evaporated, dissolved in 1 mL $CHCl_3$ and then loaded onto 2 silica gel plates (20 cm x 20 cm x 0.25 mm) which were developed in $CHCl_3$. The 2'-hydroxyubiquinone-1 band (golden yellow, R_f ~0.3) was eluted with ethanol. The yield was 30-50%.

The 2'-ANAP-Q-1 was synthesized by DR H.D. Campbell by coupling the 2'-hydroxyubiquinone-1 with ANAP using p-toluenesulphonyl chloride as a catalyst. [1-^{14}C]-ANAP was also coupled with 2'-hydroxyubiquinone-1 using the same method to yield [^{14}C]-2'-ANAP-Q-1 with a specific activity of 3 x 10^6 dpm/µmole. The overall scheme for the synthesis of the 2'-arylazidoubiquinone-1 is shown in Figure 5-3.

Covalent Radiochemical Labelling of Purified NADH:ubiquinone Oxidoreductase with [^{14}C]-2'-Arylazidoubiquinone-1

The purified enzyme (5.8 mg protein mL^{-1}) was extensively dialyzed against 5 mM potassium phosphate buffer, pH 7.5, 0.1% (w/v) cholate, 1 mM EDTA, 1 mM 2-mercaptoethanol and 20 µM FAD. 2 mL of the dialyzed enzyme was placed into a 3 mL volume stoppered quartz cuvette and after the addition of 15 µL of an ethanolic solution of

[^{14}C]-2'-arylazidoubiquinone-1 (61 mM, specific activity 3 x 10^6 dpm/µmole) was irradiated at 4°C for 3 minutes as described below. The cuvette was held 20 cm from the ultraviolet light source (C-63 mineralight, 302 nm wavelength; Ultra-Violet products, Inc.) on its side. During irradiation the cuvette was shaken slightly to mix. After addition of a further 15 µL of [^{14}C]-2'-arylazidoubiquinone-1 irradiation was continued for 3 minutes. Finally, a further 20 µL aliquot of [^{14}C]-2'-arylazidoubiquinone-1 was added and the cuvette irradiated for 6 minutes. Under these conditions the cuvette was maintained at 4°C and only a slight loss of NADH:ubiquinone-1 oxidoreductase activity was recorded (>80% activity maintained).

After irradiation in the presence of [^{14}C]-2'-arylazidoubiquinone-1 the enzyme was precipitated and washed 3 times with acetone (Poulis et al., 1981) and the pellet was dried under vacuum. The [^{14}C]-2'-arylazidoubiquinone-1 was soluble in acetone and free label was recovered in the acetone supernatant. The dried pellet was dissolved in 0.7 mL of 5 mM potassium phosphate buffer, pH 7.5, containing 10% (w/v) SDS and 5 µL of 2-mercaptoethanol. The pellet did not readily dissolve at room temperature so it was placed in a boiling water bath for 10 minutes.

The extent of covalent labelling was assessed by loading the sample onto a column (25 x 1.6 cm) of Sephacryl S-300 equilibrated with 5 mM potassium phosphate buffer, pH 7.5, containing 1 mM EDTA, 2% (w/v) SDS and 0.1% (v/v) 2-mercaptoethanol. This gel filtration procedure has been shown to completely separate endogenous FAD, phospholipid and ubiquinone-8 from the enzyme polypeptide (Jaworowski et al., 1981b; Campbell, unpublished).

Separation and Characterization of the [^{14}C]-Labelled-Polypeptides produced by BNPS-skatole Cleavage at Trp Residues

After cleavage of the [^{14}C]-labelled-polypeptide with BNPS-skatole according to procedures reported by Omenn et al. (1970) and Fontana (1972), the peptide mixture was lyophilized and dissolved in sample buffer (Laemmli, 1970) for SDS-polyacrylamide gel electrophoresis (see Chapter 4).

The cleaved peptide mixture in sample buffer was separated by SDS-polyacrylamide (12.5% w/v) slab gel electrophoresis. Four preparative slab gels were run and each of the bands (numbered 1 to 6) shown in Figure 5-11 were cut out with a razor blade (see Results and Discussion). Each preparative gel was loaded with material equivalent to ~1 mg of protein prior to cleavage. These preparative gels were not fixed in trichloroacetic acid before staining. Preparative gels were briefly stained (10 minutes) with Coomassie Blue at room temperature and destained as previously described (Chapter 4). The gel slices were eluted from the acrylamide gel by the addition of 2 mL of elution buffer (0.05 M NH_4HCO_3, 1.0% (w/v) SDS and 1.0% (v/v) 2-mercaptoethanol) and incubated at 37°C for 24 hours (Weber, Pringle and Osborn 1972). After 24 hours the buffer was removed and 2 mL of fresh buffer was added. After 48 hours the procedure was repeated. The elution procedure was demonstrated to be complete by acid hydrolysis of the eluted gel slices followed by amino acid analysis and liquid scintillation spectrometry.

The eluted peptide samples were then extensively dialyzed (12 hours) against four changes of 2 L buffer composed of 0.05 M NH_4HCO_3 and 0.1% (w/v) SDS. After dialysis the samples were placed in round bottomed flasks and freeze dried for 16 hours. The flocculent dried

material (still blue from the presence of dye) was dissolved in 3.2 mL of distilled water. 1.4 mL was added to 10 mL of scintillation cocktail and its radioactivity determined. A further 1.4 mL was used for amino acid analysis and to accurately determine the protein concentration.

Effect of added Ubiquinone-1 on the Extent of Labelling with [^{14}C]-2'-Arylazidoubiquinone-1

With the aim of determining the effect of added ubiquinone-1 on the extent of labelling by [^{14}C]-2'-arylazidoubiquinone-1 two photoirradiation experiments were performed. One experiment was performed in the presence of added ubiquinone-1 and the other in its absence. The experimental procedure used was as follows: 300 µL of concentrated enzyme (7.9 mg mL^{-1} protein) was added to a 0.5 mL volume quartz cuvette. 5 µL of a 10 mM ethanolic solution of [^{14}C]-2'-arylazidoubiquinone-1 was added and irradiated for 20 minutes under conditions outlined in the preceding section. This procedure was repeated a further 3 times. In the case of the ubiquinone-1 'protection' experiment 200 nmoles of ubiquinone-1 was added prior to irradiation (final concentration 0.6 mM >> K_m for ubiquinone-1). The labelled protein was acetone precipitated 3 times with 2 mL acetone, desiccated under vacuum for 30 minutes and then dissolved in 250 µL of 5 mM potassium phosphate buffer, pH 7.5, 5% (w/v) SDS, 1 mM EDTA and 0.1% (v/v) 2-mercaptoethanol. The dissolved sample was then loaded onto a 5 mL volume Sephacryl-300 column equilibrated with 5 mM potassium phosphate buffer, pH 7.5, 2% (w/v) SDS, 1 mM EDTA, and 0.1% (v/v) 2-mercaptoethanol. 50 Fractions of ~90 µL volume were collected and 25 µL of each fraction was subjected to liquid scintillation spectrometry.

RESULTS AND DISCUSSION

Synthesis of a Novel Photoaffinity Analogue of Ubiquinone

In order to be able to use the ANAP reporter group and to employ the strategy devised by Guillory and Chen (1977) for linking radiolabelled ANAP to NADH analogues it was necessary to introduce an esterifiable group into the ubiquinone sidechain. It was decided to attempt to synthesize 2'-hydroxyubiquinone-1. A hydroxyl group was introduced into the double bond of the isoprene sidechain of ubiquinone-1 by hydroboration followed by oxidation (see Figure 5-3 and Materials and Methods).

Characterization of 2'-Hydroxyubiquinone-1

Since the 2'-hydroxyubiquinone-1 was a new compound it was characterized in detail. As expected the purified 2'-hydroxyubiquinone-1 was more hydrophilic than ubiquinone-1 as shown by its reduced R_f on TLC plates developed in $CHCl_3$ (2'-hydroxyubiquinone-1, R_f ~0.3; ubiquinone-1, R_f ~0.8).

The ultraviolet absorption spectra of the oxidized and $NaBH_4$ reduced 2'-hydroxyubiquinone-1 were consistent with its benzoquinone ring structure (Figure 5-4). The λ_{max} (279 nm) was slightly shifted from the λ_{max} of ubiquinone-1 (275 nm). The spectrum showed typical borohydride reducibility (Crane and Barr, 1971) to yield the spectrum of the ubiquinol (λ_{max} = 290nm). The ultraviolet absorption spectra indicated that the ubiquinone ring structure and its substituents had been unaltered during the synthesis. The concentration of 2'-hydroxyubiquinone-1 was determined using the difference spectra of the oxidized/reduced forms at 279 nm ($\Delta\varepsilon$ = 12500 $M^{-1}cm^{-1}$ used as for ubiquinone-1; Crane and Barr, 1971).

Figure 5-3. The Synthesis of 2'-Arylazidoubiquinone-1

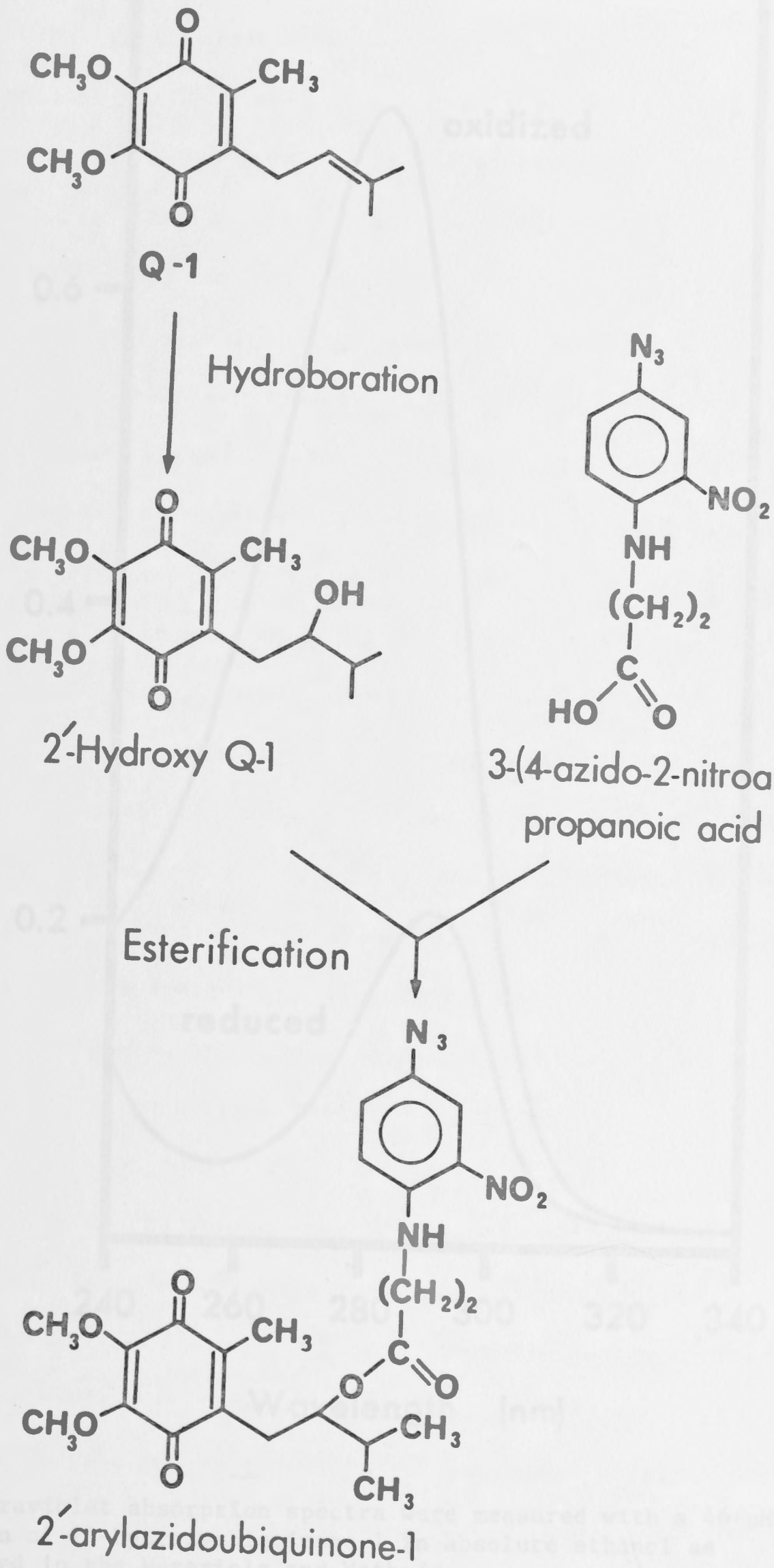
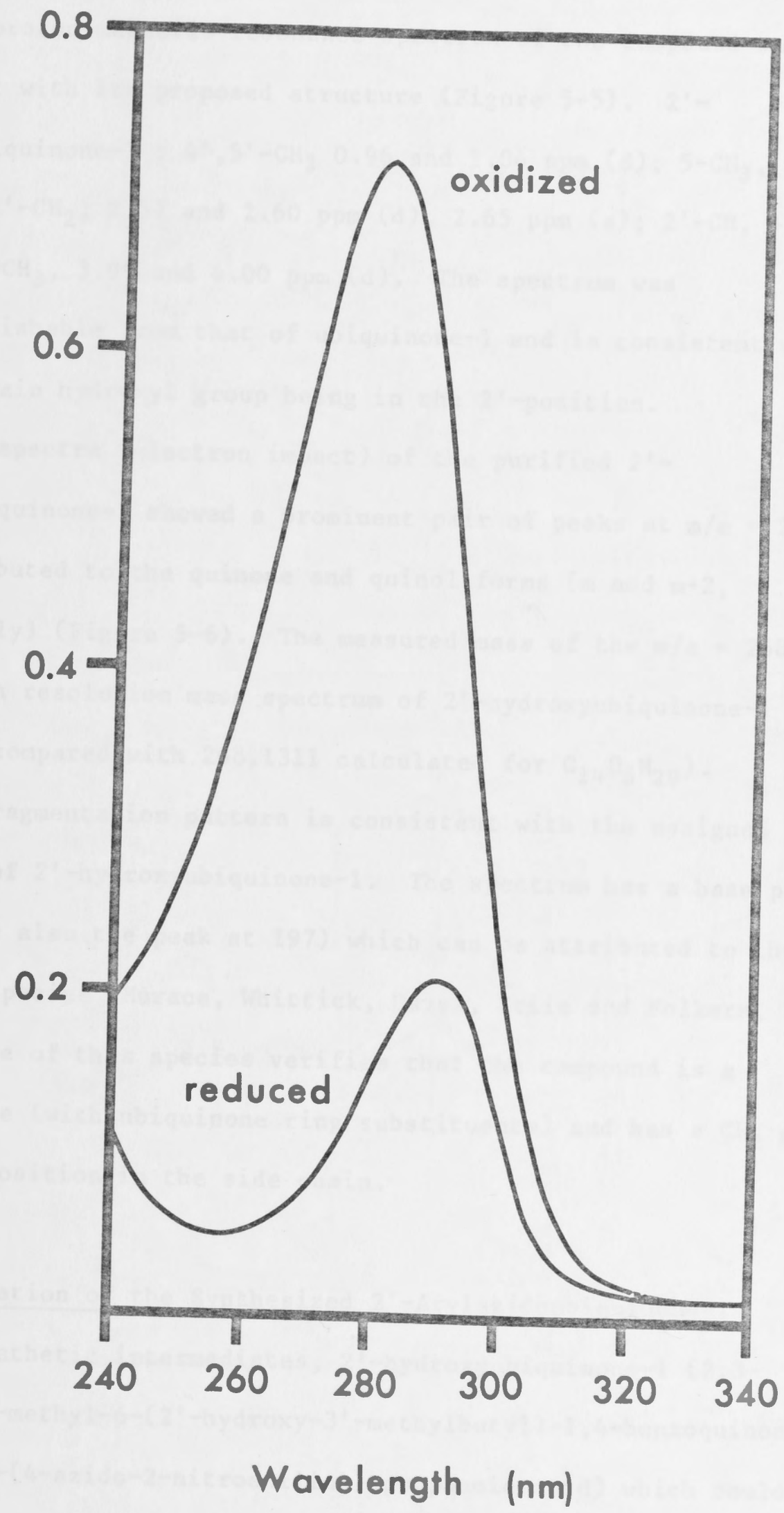


Figure 5-4. Ultraviolet Absorption Spectra of 2'-Hydroxyubiquinone



The ultraviolet absorption spectra were measured with a 46 μM solution of 2'-hydroxyubiquinone-1 in absolute ethanol as described in the Materials and Methods.

The proton magnetic resonance spectrum of the compound was also consistent with its proposed structure (Figure 5-5). 2'-Hydroxyubiquinone-1 : 4',5'-CH_3 0.96 and 1.04 ppm (d); 5-CH_3, 2.06 ppm (s); 1'-CH_2, 2.57 and 2.60 ppm (d), 2.65 ppm (s); 2'-CH, 3.46 ppm (m); 2,3-OCH_3, 3.99 and 4.00 ppm (d). The spectrum was indistinguishable from that of ubiquinone-1 and is consistent with the sidechain hydroxyl group being in the 2'-position.

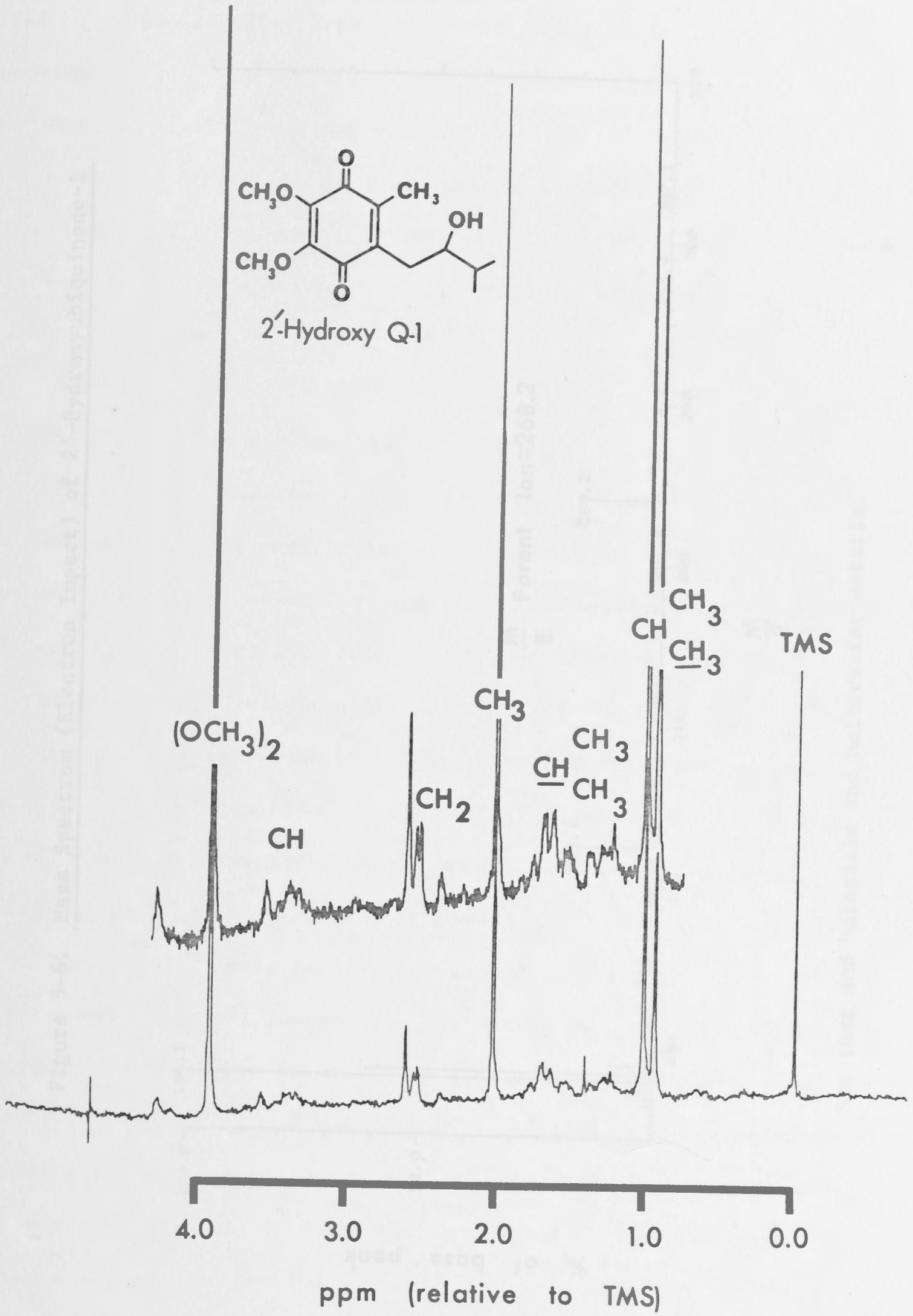
Mass spectra (electron impact) of the purified 2'-hydroxyubiquinone-1 showed a prominent pair of peaks at m/e = 268 and 270, attributed to the quinone and quinol forms (m and m+2, respectively) (Figure 5-6). The measured mass of the m/e = 268 peak in the high resolution mass spectrum of 2'-hydroxyubiquinone-1 was 268.1306 (compared with 268.1311 calculated for $C_{14}O_5H_{20}$).

The fragmentation pattern is consistent with the assigned structure of 2'-hydroxyubiquinone-1. The spectrum has a base peak of 196.1 (note also the peak at 197) which can be attributed to the benzylium species (Muraca, Whittick, Daves, Friis and Folkers, 1967). The presence of this species verifies that the compound is a benzoquinone (with ubiquinone ring substituents) and has a CH_2 group at the 1'-position in the side chain.

Characterization of the Synthesized 2'-Arylazidoubiquinone-1

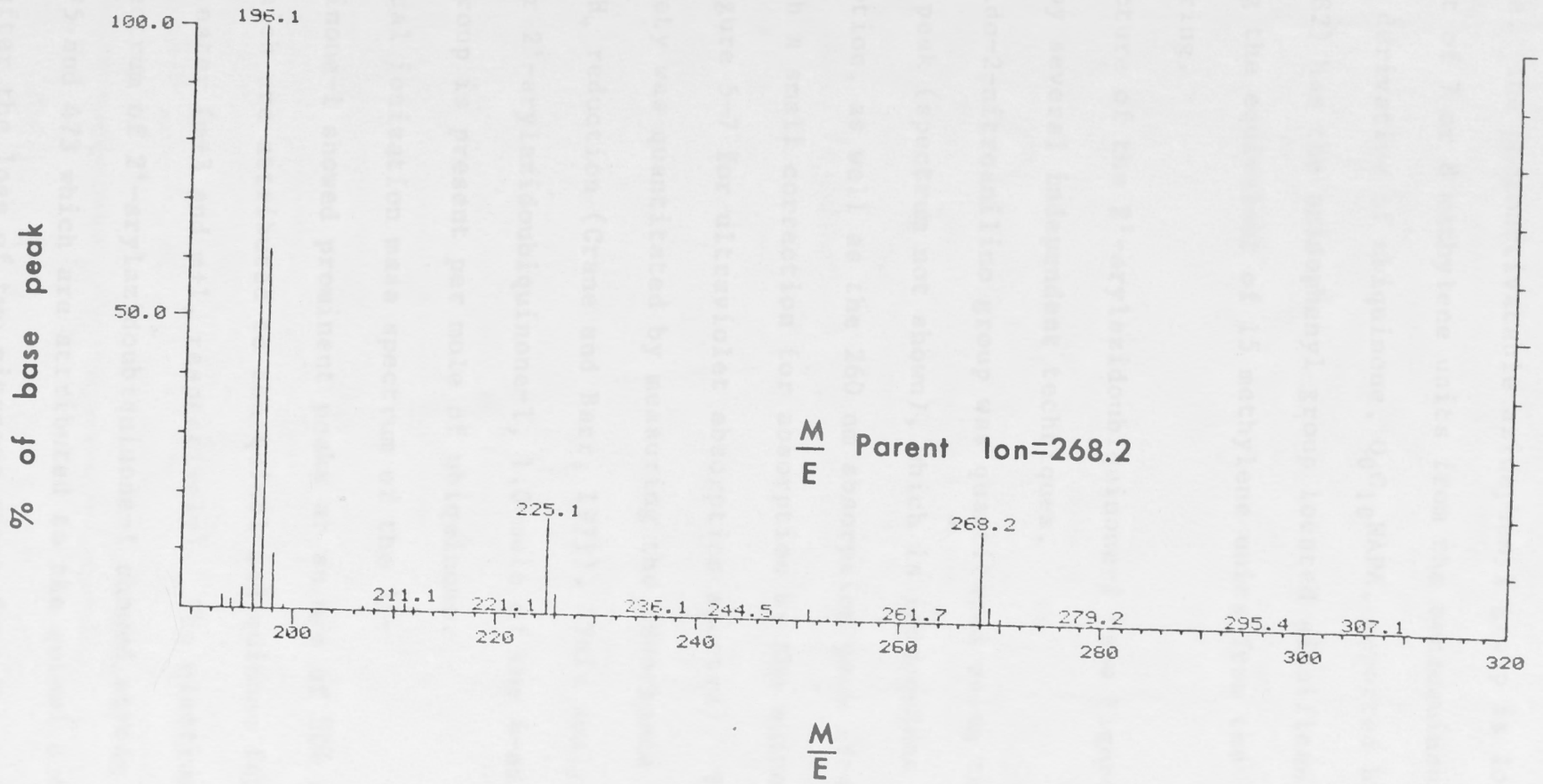
The synthetic intermediates, 2'-hydroxyubiquinone-1 (2,3-dimethoxy-5-methyl-6-(2'-hydroxy-3'-methylbutyl)-1,4-benzoquinone) and ANAP (3-(4-azido-2-nitroanilino) propanoic acid) which could be radiochemically labelled, were each synthesized and purified and then coupled via an esterification reaction. The product, named for simplicity 2'-arylazidoubiquinone-1 or 2'-ANAP-Q-1, was purified and

Figure 5-5. Proton Magnetic Resonance Spectrum of 2'-Hydroxyubiquinone-1



Approximately 1 mg of sample was dissolved in 0.5 mL $CDCl_3$. Chemical shifts are expressed relative to the internal standard tetramethylsilane (TMS) (see Materials and Methods for further details).

Figure 5-6. Mass Spectrum (Electron Impact) of 2'-Hydroxyubiquinone-1



See text and Materials and Methods for details.

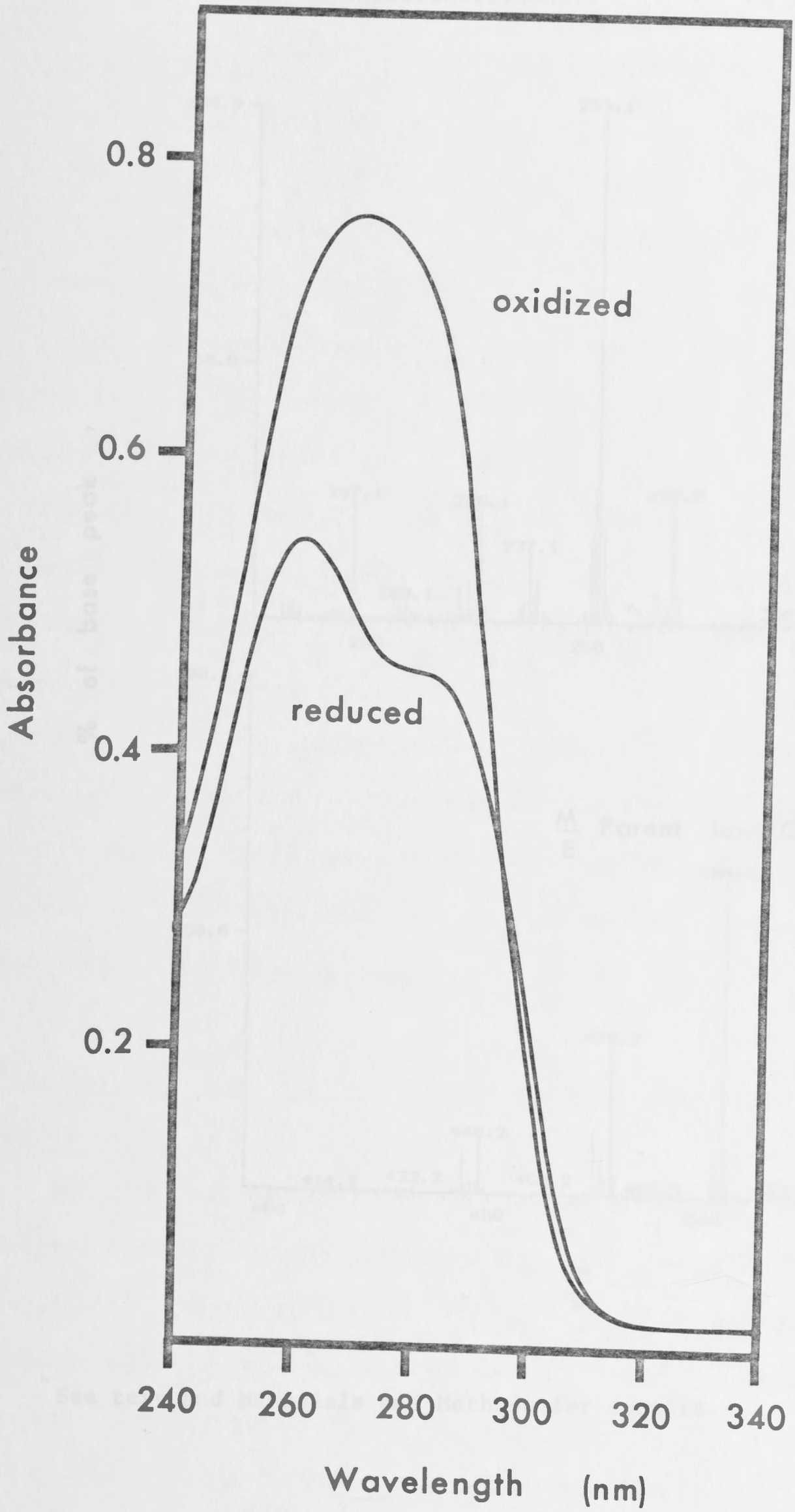
used to radiochemically label the purified NADH:ubiquinone oxidoreductase. The photoactivatable azidophenyl group is located the equivalent of 7 or 8 methylene units from the benzoquinone ring. The analogous derivative of ubiquinone, Q_0C_{10}NAPA, reported by Yu and Yu (1980b; 1982) has the azidophenyl group located significantly more distant, being the equivalent of 15 methylene units from the benzoquinone ring.

The structure of the 2'-arylazidoubiquinone-1 (see Figure 5-3) was verified by several independent techniques.

The 4-azido-2-nitroanilino group was quantitated using the 450 nm absorption peak (spectrum not shown), which is independent of the quinone absorption, as well as the 260 nm absorption peak after $NaBH_4$ reduction, with a small correction for absorption by the quinol moiety (see Figure 5-7 for ultraviolet absorption spectra). The ubiquinone moiety was quantitated by measuring the absorbance change at A_{279} on $NaBH_4$ reduction (Crane and Barr, 1971). This analysis showed that for 2'-arylazidoubiquinone-1, 1.0 mole of the 4-azido-2-nitroanilino group is present per mole of ubiquinone.

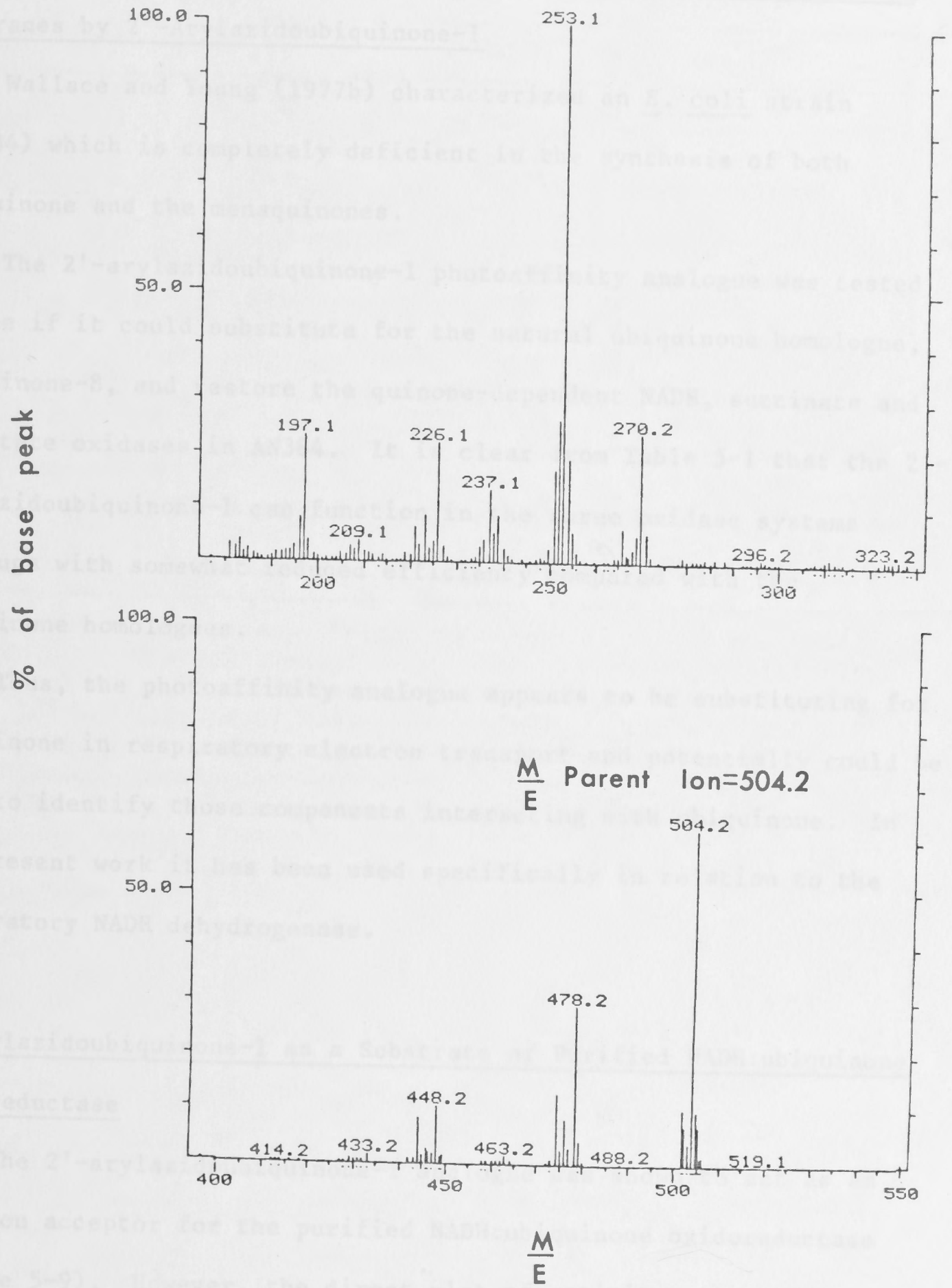
The chemical ionization mass spectrum of the 2'-arylazidoubiquinone-1 showed prominent peaks at an m/e of 504 and 502 (Figure 5-8) which are attributed to the quinol and quinone forms of the protonated ester (m+3 and m+1, respectively). The electron impact mass spectrum of 2'-arylazidoubiquinone-1 showed strong peaks at an m/e of 475 and 473 which are attributed to the quinol and quinone forms after the loss of two nitrogen atoms from the azido group.

Figure 5-7. Ultraviolet Absorption Spectra of 2'-Arylazidoubiquinone-1



The ultraviolet absorption spectra were measured with a 22 μM solution of 2'-arylazidoubiquinone-1 in absolute ethanol as described in the Materials and Methods.

Figure 5-8. Mass Spectrum (Chemical Ionization) of 2'-Arylazidoubiquinone-1



See text and Materials and Methods for details.

Reconstitution of NADH, Succinate and D-Lactate Oxidase in *ubi men* Membranes by 2'-Arylazidoubiquinone-1

Wallace and Young (1977b) characterized an *E. coli* strain (AN384) which is completely deficient in the synthesis of both ubiquinone and the menaquinones.

The 2'-arylazidoubiquinone-1 photoaffinity analogue was tested to see if it could substitute for the natural ubiquinone homologue, ubiquinone-8, and restore the quinone-dependent NADH, succinate and D-lactate oxidases in AN384. It is clear from Table 5-1 that the 2'-arylazidoubiquinone-1 can function in the three oxidase systems although with somewhat reduced efficiency compared with the ubiquinone homologues.

Thus, the photoaffinity analogue appears to be substituting for ubiquinone in respiratory electron transport and potentially could be used to identify those components interacting with ubiquinone. In the present work it has been used specifically in relation to the respiratory NADH dehydrogenase.

2'-Arylazidoubiquinone-1 as a Substrate of Purified NADH:ubiquinone Oxidoreductase

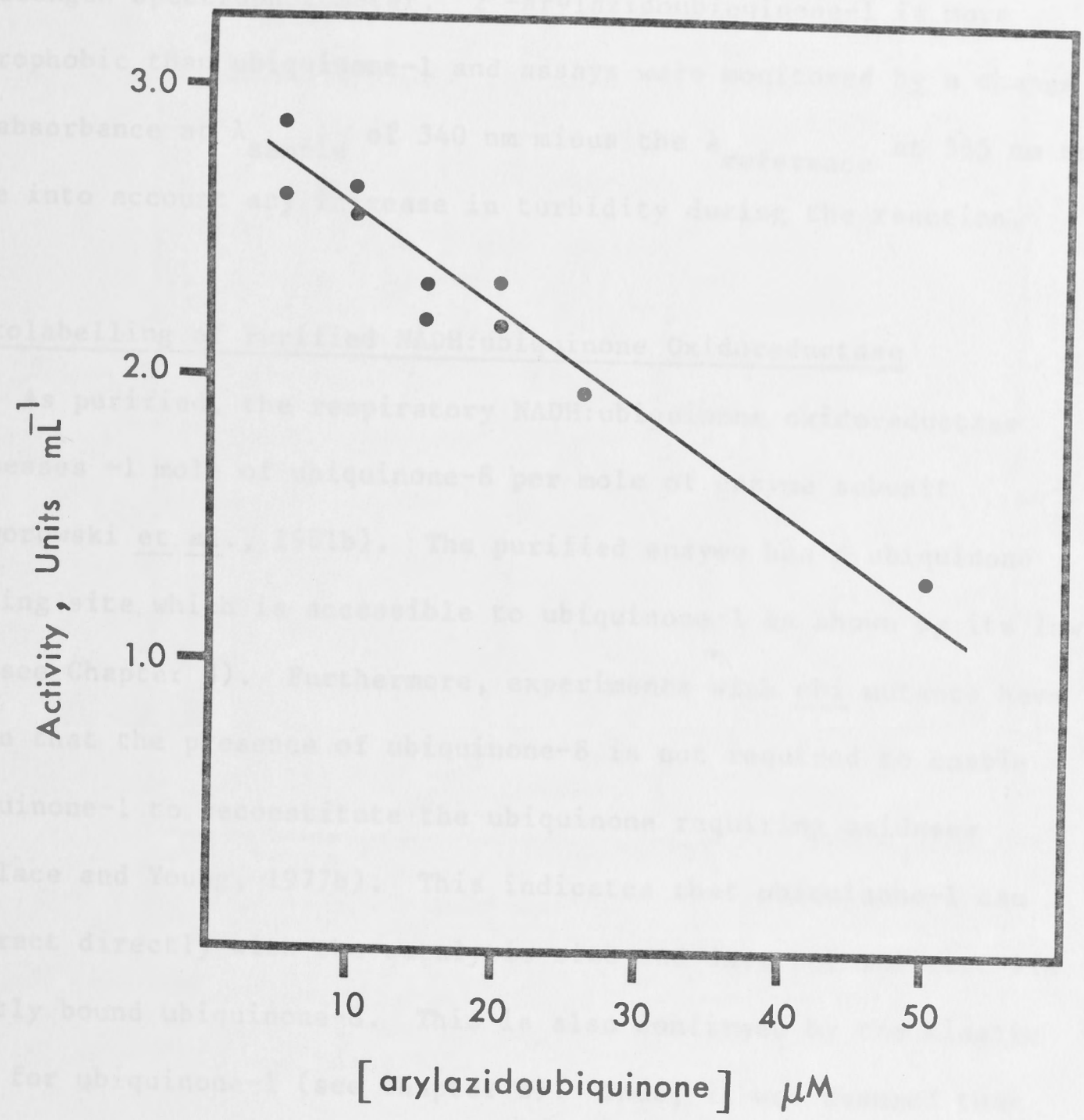
The 2'-arylazidoubiquinone-1 analogue was shown to act as an electron acceptor for the purified NADH:ubiquinone oxidoreductase (Figure 5-9). However, the direct plot of activity verses substrate concentration does not exhibit hyperbolic kinetics. The nature of the plot suggests some form of substrate inhibition. The kinetics of the reaction were not further characterized. The highest rate recorded (2.8 units mL^{-1}) was ~30% of that for ubiquinone-1 (at 50 μM concentration). The assays were performed with an Aminco Chance dual

Table 5-1. Restoration of Oxidase Activities in *ubi men* Membrane Vesicles by Ubiquinone Analogues

Membrane vesicles	Quinone added	Oxidase (% Q-3)		
		NADH	Succinate	D-Lactate
AN384 (*ubiA menA*)	Q-3	100	100	100
	Q-1	71	93	100
	2'-ANAP-Q-1	65	71	53
	Nil	4	4	8
AN387	Q-3	100	100	100
	Q-1	64	104	75
	2'-ANAP-Q-1	76	100	88
	Nil	76	96	81

The oxidase assays were performed as described in the Materials and Methods. Rates are expressed as a percentage of the stimulated rates with Q-3 added. The actual rates were as follows: AN384, 2290, 207 and 112; AN387, 1036, 340 and 47; ng atoms O $min^{-1}mg^{-1}$ protein for NADH, succinate and D-lactate oxidases, respectively. The ubiquinone analogues were added to give a final concentration of 20 μM. AN387 oxidase levels are equivalent to wild type levels.

Figure 5-9. Direct Plot for the Reduction of 2'-Arylazidoubiquinone-1 by Purified NADH:ubiquinone Oxidoreductase



Assays were performed as described in the text and in the Materials and Methods (Chapter 3). 0.86 μg protein was added per assay.

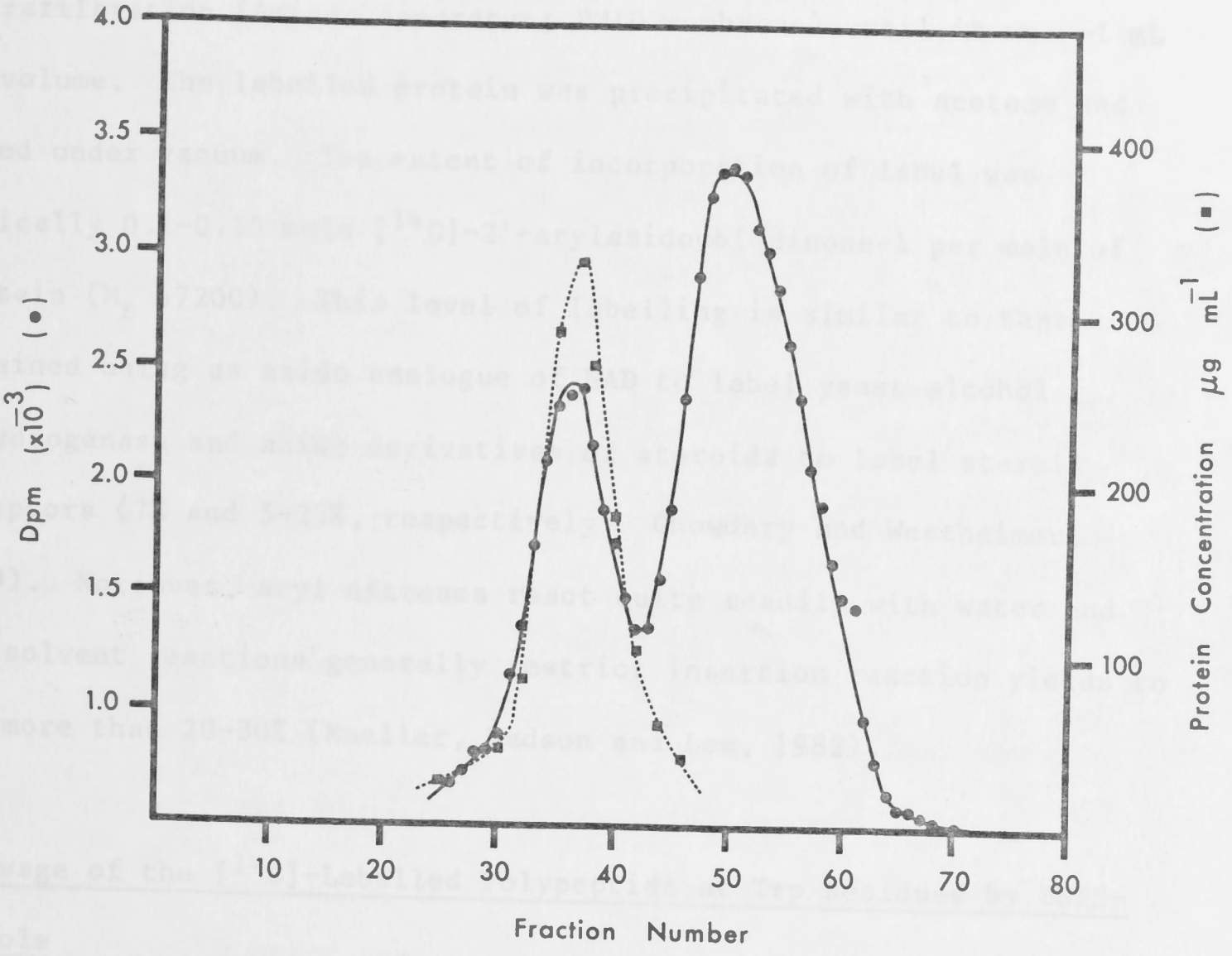
wavelength spectrophotometer. 2'-Arylazidoubiquinone-1 is more hydrophobic than ubiquinone-1 and assays were monitored by a change in absorbance at λ_{sample} of 340 nm minus the $\lambda_{reference}$ at 395 nm to take into account any increase in turbidity during the reaction.

Photolabelling of Purified NADH:ubiquinone Oxidoreductase

As purified, the respiratory NADH:ubiquinone oxidoreductase possesses ~1 mole of ubiquinone-8 per mole of enzyme subunit (Jaworowski et al., 1981b). The purified enzyme has a ubiquinone binding site which is accessible to ubiquinone-1 as shown by its low K_m (see Chapter 3). Furthermore, experiments with ubi mutants have shown that the presence of ubiquinone-8 is not required to enable ubiquinone-1 to reconstitute the ubiquinone requiring oxidases (Wallace and Young, 1977b). This indicates that ubiquinone-1 can interact directly with the catalytic site and does not interact via tightly bound ubiquinone-8. This is also confirmed by the kinetic data for ubiquinone-1 (see Chapter 3). Thus, it was assumed that ubiquinone-1 derivatives such as [^{14}C]-2'-arylazidoubiquinone-1 would have access to the catalytic site for the purposes of photoaffinity labelling.

[^{14}C]-2'-arylazidoubiquinone-1 was irradiated in the presence of purified enzyme as described in the Materials and Methods section. The [^{14}C]-labelled protein was separated from unattached [^{14}C]-label by chomatography on a Sephacryl S-300 column. Two peaks of radioactivity were observed, the first of which was coincident with the protein peak (Figure 5-10). The second radioactive peak was attributed to labelled phospholipid or unreacted [^{14}C]-2'-arylazidoubiquinone-1.

Figure 5-10. Sephacryl S-300 Chromatogram of [^{14}C]-Labelled Polypeptide



The conditions of chromatography are presented in the text and in the Materials and Methods.

The radioactive protein peak was pooled and concentrated by ultrafiltration (Amicon apparatus; PM10 membrane) until it was ~1 mL in volume. The labelled protein was precipitated with acetone and dried under vacuum. The extent of incorporation of label was typically 0.1-0.15 mole [^{14}C]-2'-arylazidoubiquinone-1 per mole of protein (M_r 47200). This level of labelling is similar to that obtained using an azido analogue of NAD to label yeast alcohol dehydrogenase and azido derivatives of steroids to label steroid receptors (7% and 5-25%, respectively; Chowdhry and Westheimer, 1979). Moreover, aryl nitrenes react quite readily with water and the solvent reactions generally restrict insertion reaction yields to not more than 20-30% (Mueller, Hudson and Lee, 1982).

Cleavage of the [^{14}C]-Labelled Polypeptide at Trp Residues by BNPS-Skatole

With the aim of establishing the predominant site of label attachment, the [^{14}C]-labelled polypeptide was cleaved at tryptophan residues using BNPS-skatole. The cleavage fragments produced were separated by preparative SDS-polyacrylamide gel electrophoresis, peptide staining bands were cut out and the peptides eluted from the gel slices and characterized.

There are two tryptophan residues at positions 47 and 272 in the sequence of the enzyme. Cleavage at these points would theoretically lead to the production of three polypeptides: residues 2-47 (mol wt 5000), residues 48-272 (mol wt 24500) and residues 273-434 (mol wt 17900) (see Chapter 2 for sequence). Partial cleavage would lead to the production of polypeptides composed of residues 2-272 (mol wt 29400) and residues 48-434 (mol wt 42300).

Figure 5-11 shows an analytical gel of the BNPS-skatole cleavage fragments. The numbers illustrated in Figure 5-11 represent the six major peptide species produced. A densitometric trace (see Chapter 3, Materials and Methods) of the cleavage products was performed (Figure 5-11). Using this procedure it was calculated that the six numbered peptides constituted ~58% of the total protein loaded on the gel. The peptide bands designated 1 to 6 were processed as described in the Materials and Methods. The major peptide band nearest band 3 was not processed since it was diffuse and appeared heterogeneous.

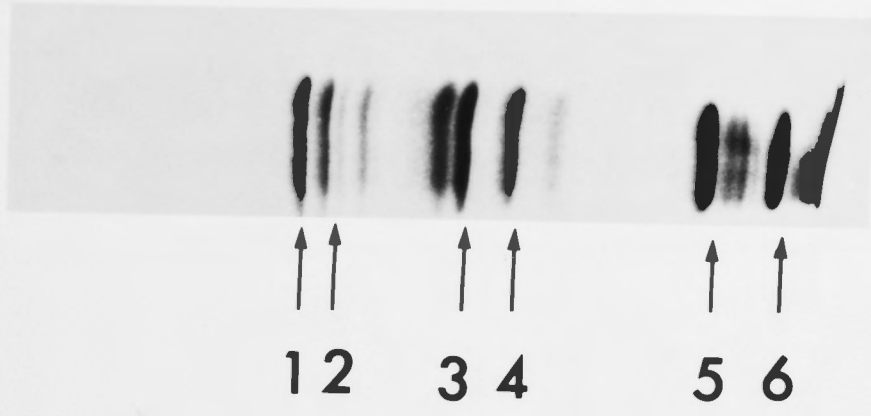
The apparent M_r values for the cleavage peptides were determined by comparisons with proteins of known M_r. From the sizes of the cleavage products it was possible to assign fragments 1 to 5 in terms of the amino acid sequence (Table 5-2). These assignments were verified by amino acid compositions (Table 5-3).

It should be noted that band 6, the heavy band visible nearest the dye front, was also eluted and analysed. Since its amino acid composition did not fit any of the predicted total or partial Trp cleavage products it has not been definitely assigned and consequently is not included in Tables 5-2 and 5-3.

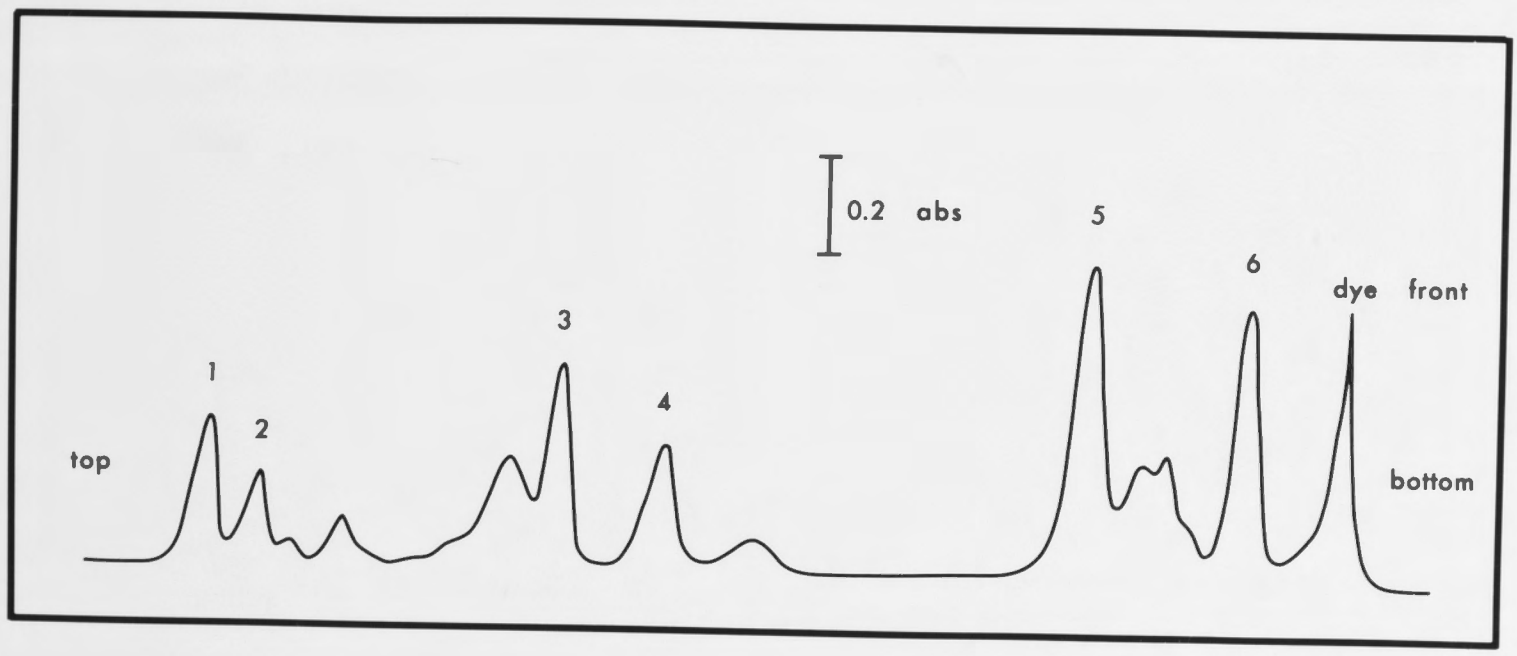
The amino acid composition analysis, protein concentration analysis and liquid scintillation spectrometry enabled the extent (dpm/nmole peptide) and the stoichiometry (mole [^{14}C]-label/mole peptide) of labelling to be calculated (Table 5-4). The results suggest that there is some apparent loss of label (~55%) during cleavage, separation and elution of the peptides 3 and 5 (ie the sum of the dpm/nmole peptide values for bands 3 and 5 is ~45% of that for the complete uncleaved polypeptide, band 1). The reason for this is

Figure 5-11. SDS-Polyacrylamide Gel Electrophoresis and Densitometric Trace of [^{14}C]-Labelled Peptides Produced by BNPS-Skatole Cleavage of [^{14}C]-Labelled-Protein

Analytical SDS
Polyacrylamide Gel



Densitometric Trace



The conditions of cleavage by BNPS-skatole and SDS-polyacrylamide gel electrophoresis are described in the text and in the Materials and Methods.

Table 5-2. Properties of Bands Isolated from [^{14}C]-2'-Arylazidoubiquinone-1 Labelled NADH: ubiquinone Oxidoreductase after Partial Cleavage at Trp Residues

Band[a]	Assignment[b]	Apparent Mol Wt[c]	Calculated Mol Wt[d]
1	2-434	47000	47200
2	48-434	43000	42300
3	2-272	31000	29400
4	48-272	26500	24500
5	273-434	18000	17900

[a] Band assigned from SDS-polyacrylamide gel electrophoresis.

[b] Residue numbering.

[c] Determined by SDS-polyacrylamide gel electrophoresis.

[d] Calculated from sequence.

Table 5-3. Amino Acid Composition of Peptides Isolated from NADH: ubiquinone Oxidoreductase After Partial Cleavage at Trp Residues

Amino Acid	Band [a] (Residues) 1 [b] (2-434)	2 (48-434)	3 (2-272)	4 (48-272)	5 (273-434)
D	41.8 (41)	41.3 (39)	26.8 (26)	25.6 (24)	14.4 (15)
T	25.6 (26)	21.6 (22)	17.1 (18)	13.5 (14)	7.7 (8)
S	21.6 (20)	20.3 (19)	12.0 (11)	10.8 (10)	7.8 (9)
E	38.2 (37)	35.1 (35)	26.2 (26)	24.2 (24)	12.0 (11)
P	15.0 (17)	10.9 (16)	8.5 (8)	8.3 (7)	6.7 (9)
G	43.1 (42)	36.9 (35)	26.8 (27)	21.0 (20)	14.1 (15)
A	39.3 (39)	35.4 (36)	25.6 (26)	22.9 (23)	12.9 (13)
V [c]	23.8 (27)	21.8 (24)	17.6 (19)	15.6 (16)	7.6 (8)
M [d]	5.6 (14)	7.0 (13)	3.7 (5)	3.8 (4)	5.1 (9)
I [c]	20.7 (26)	18.3 (23)	11.7 (14)	9.6 (11)	8.4 (12)
L	48.1 (51)	43.1 (45)	33.2 (35)	27.2 (29)	15.5 (16)
Y [d]	10.1 (12)	9.6 (12)	5.9 (6)	5.7 (6)	4.6 (6)
F	10.0 (11)	9.9 (11)	6.2 (6)	5.8 (6)	4.0 (5)
H	16.8 (17)	8.9 (14)	10.6 (12)	8.8 (9)	5.1 (5)
K	24.6 (25)	19.6 (18)	15.8 (18)	10.3 (11)	6.6 (7)
R [e]	15.1 (22)	14.2 (20)	8.8 (11)	7.1 (9)	6.8 (11)

[a] Numbering of bands is as shown in Figure 5-11. Residue numbering (inclusive) is shown in brackets. Residue 1 (M) is not present in the purified enzyme.

[b] Uncleaved enzyme.

[c] Low values attributed to slow hydrolysis.

[d] Low values attributed to oxidation.

[e] Reason for low values unknown.

Table 5-4. Localization of Labelling of the NADH:ubiquinone Oxidoreductase with [^{14}C]-2'-Arylazidoubiquinone-1

Band	Section of Polypeptide (Residue numbering)	dpm[a] / eluted peptide	nmole eluted peptide[b]	dpm / nmole peptide	dpm / nmole of 100 residues	mole [^{14}C][c] / mole polypeptide
1	2-434	264	0.66	390	90	0.13[d]
2	48-434	327	0.89	370	95	0.12
3	2-272	329	2.7	120	44	0.04
4	48-272	407	3.7	110	49	0.04
5	273-434	531	9.1	60	37	0.02

[a] The dpm values are averaged figures derived from two separate counts of 1 hour duration.

[b] These yields were calculated from the amino acid composition data using residues D, E, A and L (see Table 5-3).

[c] Calculated from the incorporation of radioactivity and the specific radioactivity of the [^{14}C]-2'-arylazidoubiquinone-1.

[d] The pooled concentrated enzyme after gel filtration (Figure 5-10) in the presence of SDS had a mole [^{14}C]/mole polypeptide stoichiometry of 0.12.

unknown. Exposure of peptides to BNPS-skatole leads to the cleavage of tryptophanyl peptide bonds (C-terminal position) and the oxidation of the indole ring (Omenn et al., 1970). If tryptophan was heavily labelled it is possible that some loss of label could occur during the BNPS-skatole cleavage procedure but this explanation seems unlikely.

As pointed out by Bayley and Knowles (1977) the exact nature of linkages formed to proteins by photolabile reagents has not been clarified. A few attempts to locate labelled amino acids have indicated that some of the linkages may be chemically labile since a considerable amount of radioactivity is lost during the analysis. For instance, significant losses (~40%) of nitrene-derived label have been shown to occur on treatment with cyanogen bromide (Fisher and Press, 1974). Fisher and Press (1974) ascertained that the loss of radioactive label represented a bound non-covalent label-lysine derivative.

The BNPS-skatole cleavage allows comparison of the extent of labelling of three regions of the protein encompassing residues 2-42, 48-272 and 273-434 (Table 5-4). The extent of labelling of the small N-terminal fragment is obtained by comparing bands 1 and 2 and also bands 3 and 4 and it seems clear that this segment carries less label than the other two bands. The middle region of the protein appears to be more highly labelled than the C-terminal segment. However, at present it is not possible to say whether the labelling of these two fragments is highly localized or not.

At this stage it was considered important to test whether excess ubiquinone-1 could specifically block labelling of the enzyme by the photoaffinity analogue.

Attempts were made to assess the effect of the addition of ubiquinone-1 on the extent of labelling with [^{14}C]-2'-arylazidoubiquinone-1. However, the extent of labelling in the presence of ubiquinone was in the same range as that obtained in its absence (~0.08-0.15 mole [^{14}C]/mole peptide). The fact that the presence of ubiquinone-1 did not markedly reduce the extent of labelling raised doubts as to the specificity of the labelling and further characterization of the positions of the label was not carried out.

Jaworowski et al. (1981b) showed that pure preparations of the NADH:ubiquinone oxidoreductase contain 70% (w/w) lipid, predominantly phosphatidylethanolamine. At present the sites of phospholipid binding on the enzyme are unknown. The added 2'-arylazidoubiquinone-1 could well be locally concentrated in the phospholipid and photolysis may lead to covalent labelling of the lipid-binding sites as well as the specific ubiquinone active site. This may explain the lack of significant protection by ubiquinone-1 of the labelling by the photoaffinity analogue.

SUMMARY

A novel photoaffinity analogue of ubiquinone has been synthesized by coupling (using p-toluenesulphonylchoride) 3-(4-azido-2-nitroanilino) propanoic acid to a ubiquinone-1 derivative containing a hydroxyl group on the sidechain. This method may prove useful in linking other carboxylic acids containing a range of reporter groups to 2'-hydroxyubiquinone-1. This route becomes more

valuable when it is considered that [^{3}H]-labelling of 2'-hydroxyubiquinone-1 can be accomplished by the use of [^{3}H]-$NaBH_4$ in the 2'-hydroxyubiquinone-1 synthesis procedure.

Using the readily available [1-^{14}C]-3-aminopropanoic acid (54.7 Ci/mole) as one of the starting materials a [^{14}C]-labelled preparation of 2'-arylazidoubiquinone-1 with high specific activity (3×10^6 dpm/μmole) was prepared with a high yield (~36% radiochemical yield).

The 2'-arylazidoubiquinone photoaffinity analogue has been shown to be capable of acting as an electron acceptor with purified enzyme at levels approaching ~30% of that for ubiquinone-1. The synthesized analogue has also been shown to be capable of restoring NADH, succinate and D-lactate oxidases in ubi men membrane vesicles, suggesting that it can substitute for ubiquinone-8 in the respiratory chain. In future the [^{14}C]-2'-arylazidoubiquinone-1 may be usefully employed in probing other sections of the respiratory chain which utilize ubiquinone.

After photolysis the [^{14}C]-2'-arylazidoubiquinone-1 was shown to be covalently attached (typically 0.1-0.15 mole label per mole polypeptide) to the purified enzyme. The enzyme was cleaved at tryptophan residues by BNPS-skatole. The labelled peptides produced were separated with high resolution by SDS-polyacrylamide gel electrophoresis, eluted from the gel and analyzed. Complete and partial cleavage peptides were isolated which encompassed the complete sequence. The peptides were identified by their migration upon SDS-polyacrylamide gel electrophoresis and their apparent molecular weights calculated. The assigned apparent molecular weights and sequence were verified by amino acid analysis.

The results suggested that the middle region of the protein appeared to be more heavily labelled than the C-terminal region which was more labelled than the N-terminal region. However, the interpretation of the labelling data was made more difficult because of a significant loss of label (~55%) during the cleavage, separation and elution of the peptides. Furthermore, photolysis experiments with and without ubiquinone-1 present showed that the presence of ubiquinone-1 did not lead to a decrease in the extent of labelling. The presence of relatively high levels of phospholipid on the enzyme may concentrate the photoaffinity reagent and lead to the labelling of phospholipid binding sites as well as residues in the region of the catalytic site.

Chapter 6

The Partial DNA Sequence of the Cloned *Dld* Gene

INTRODUCTION

The membrane-bound D-lactate dehydrogenase of E. coli has been solubilized and purified to homogeneity (Kohn and Kaback, 1973; Futai 1973). The enzyme possesses approximately 1 mole of FAD per mole of enzyme and has a reported molecular weight of 75000 ± 7% (Kohn and Kaback, 1973; Futai, 1973). Furthermore, the purified respiratory D-lactate dehydrogenase has been shown to reconstitute the D-lactate oxidase and D-lactate-dependent active transport activities of membrane vesicles prepared from a dld mutant strain of E. coli ML 308-222 (Short, Kaback and Kohn, 1974).

Wallace and Young (1977b) showed that the D-lactate oxidase system required a quinone for activity (see Chapters 1 and 3 for further discussion).

Young and Wallace (1976) isolated a strain of E. coli carrying a point mutation affecting the respiratory NADH dehydrogenase (ndh mutant; see Chapter 1 for further details). During attempts to clone the gene for the respiratory NADH dehydrogenase of E. coli from chromosomal DNA two hybrid plasmids were isolated (Young et al., 1978). One of the plasmids, pIY1, derived from EcoRI digested chromosomal DNA was studied by Young and colleagues and shown to possess the gene coding for the NADH dehydrogenase (ibid.). Plasmid pIY1 when transformed into an ndh mutant, IY12 (thi his ilv trp rpsL ndh), was shown not only to complement the growth of the ndh mutant strain on mannitol-minimal medium but also to amplify the NADH dehydrogenase to 8-10 times the normal level (Young et al., 1978). The other plasmid, pIY2, partially complemented the ndh mutant in terms of its ability to grow on mannitol-minimal medium but did not restore the NADH dehydrogenase levels in respiratory membranes.

Plasmid pIY2 was constructed of follows: HindIII was used to cleave a preparation of E. coli chromosomal DNA and the fragments produced were ligated into HindIII digested pGM706 (Hamer and Thomas, 1976). The ligated hybrid plasmids were transformed into an intermediate F^+ strain and then transferred by F-mobilization to IY12 (F^-,ndh rpsL). The cells were then selected for the complementation of ndh (Young et al., 1978). Electrophoresis of the HindIII digested pIY2 on an agarose gel indicated that it carries a 4.6 Mdal (~6.9 kb) DNA fragment. Young, Jaworowski and Poulis (1982) demonstrated that cells carrying pIY2, in contrast to cells carrying the parent plasmid pGM706, overproduce the respiratory D-lactate dehydrogenase in their cell membranes by 15-20 fold with negligible activity appearing in the cytoplasm (ibid.). The pIY2 plasmid was further characterized by Santos, Kung, Young and Kaback (1982). Santos et al. (1982) showed that pIY2 expressed the dld gene either in vivo with transformed minicells or in vitro with a fractionated transcription/translation system. The product formed is specifically immunoprecipitated by γ-globulin prepared against the purified enzyme and comigrates with purified D-lactate dehydrogenase on SDS-polyacrylamide gels (ibid.). Therefore, it is clear that the plasmid pIY2 carries the structural gene coding for the respiratory D-lactate dehydrogenase of E. coli. It is believed that the considerable elevation of D-lactate oxidase which occurs in the ndh mutant carrying pIY2, provides an alternative route for the reoxidation of NADH and that this is responsible for the partial restoration of the growth of the ndh mutant (Young et al., 1982).

The cloning of the respiratory D-lactate dehydrogenase provides the opportunity to determine the primary sequence of the protein via DNA sequencing. Since the D-lactate oxidase is quinone-dependent (Wallace and Young, 1977b), and partially purified D-lactate dehydrogenase reduces ubiquinone-1 at high catalytic rates (I.G. Young, unpublished), it seems likely that the respiratory D-lactate dehydrogenase will also possess a ubiquinone binding site analogous to that of the respiratory NADH dehydrogenase. Furthermore, the dld protein also must possess an FAD binding site. The determination of the complete primary sequence of the dld protein would provide a basis for examining its evolutionary relationship with the ndh protein as well as with a number of soluble D-lactate dehydrogenases. It could also assist with the identification of the ubiquinone binding sites of the two respiratory dehydrogenases. For these reasons the determination of the DNA sequence of the dld gene was initiated.

MATERIALS AND METHODS

Many of the experimental procedures used in this chapter were described previously in the Materials and Methods section of Chapter 2. New procedures or significant variations are described below.

Strains and Plasmids

Strain IY36 is an E. coli K12 derivative defective in the ndh gene (Young and Wallace, 1976). Strain IY36 carries the plasmid pIY2 which contains an insert encoding the dld gene (Young et al.,

1982). The pIY2 plasmid DNA was isolated as described in the Materials and Methods section of Chapter 2.

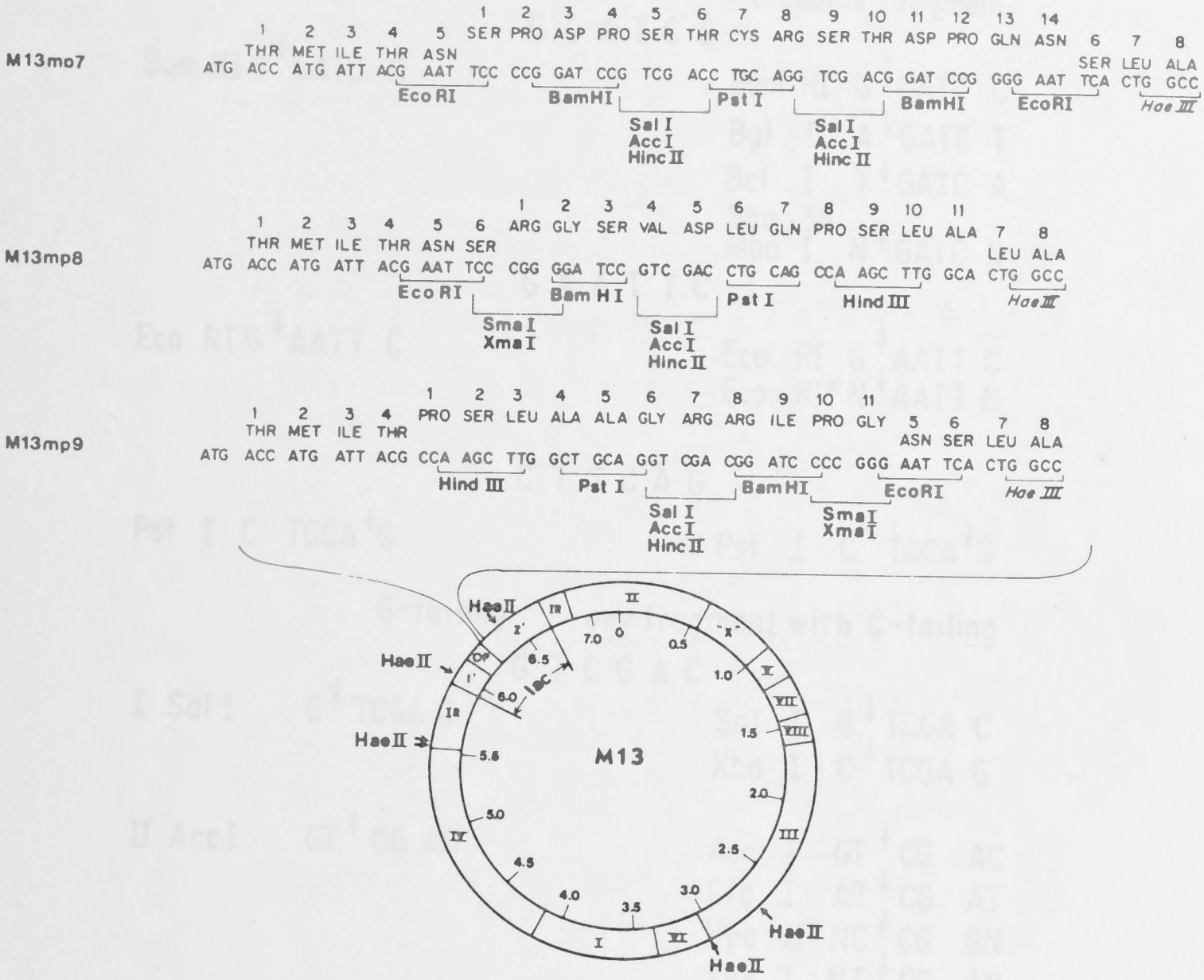
E. coli JM103 has been described previously (Messing *et al.*, 1981) and has the following reported genotype: *Δlacpro thi strA supE endA sbcB15 hsdR4* F'*traD36 proAB lacI*^{q} Z*Δ*M15. JM103 was used as the recipient cell in transfections with M13mp8 (Messing and Vieira, 1982; see Introduction). Recently, it has been reported (Focus Vol 5 No.1, Mar 1983, Bethesda Research Laboratories, Inc. publication) that the strain JM103 has some notable drawbacks. These include: JM103 may not be *hsdR4* (ie JM103 is not deficient in the K12 restriction system as initially reported by Dr J. Messing) and JM103 contains a cryptic P1 lysogen which may be responsible for observed low efficiencies of transformation or M13 phage transfection with JM103 compared to other *E. coli* host strains. Neither of these drawbacks were a problem in the context of the present work.

M13 Vectors for DNA Sequencing

The M13 vector used was M13mp8. This is one of several new cloning vectors constructed by Messing and his colleagues (Messing *et al.*, 1981; Messing and Vieira, 1982). These vectors carry multipurpose cloning sites allowing a wide variety of different fragments to be cloned (Figures 6-1 and 6-2).

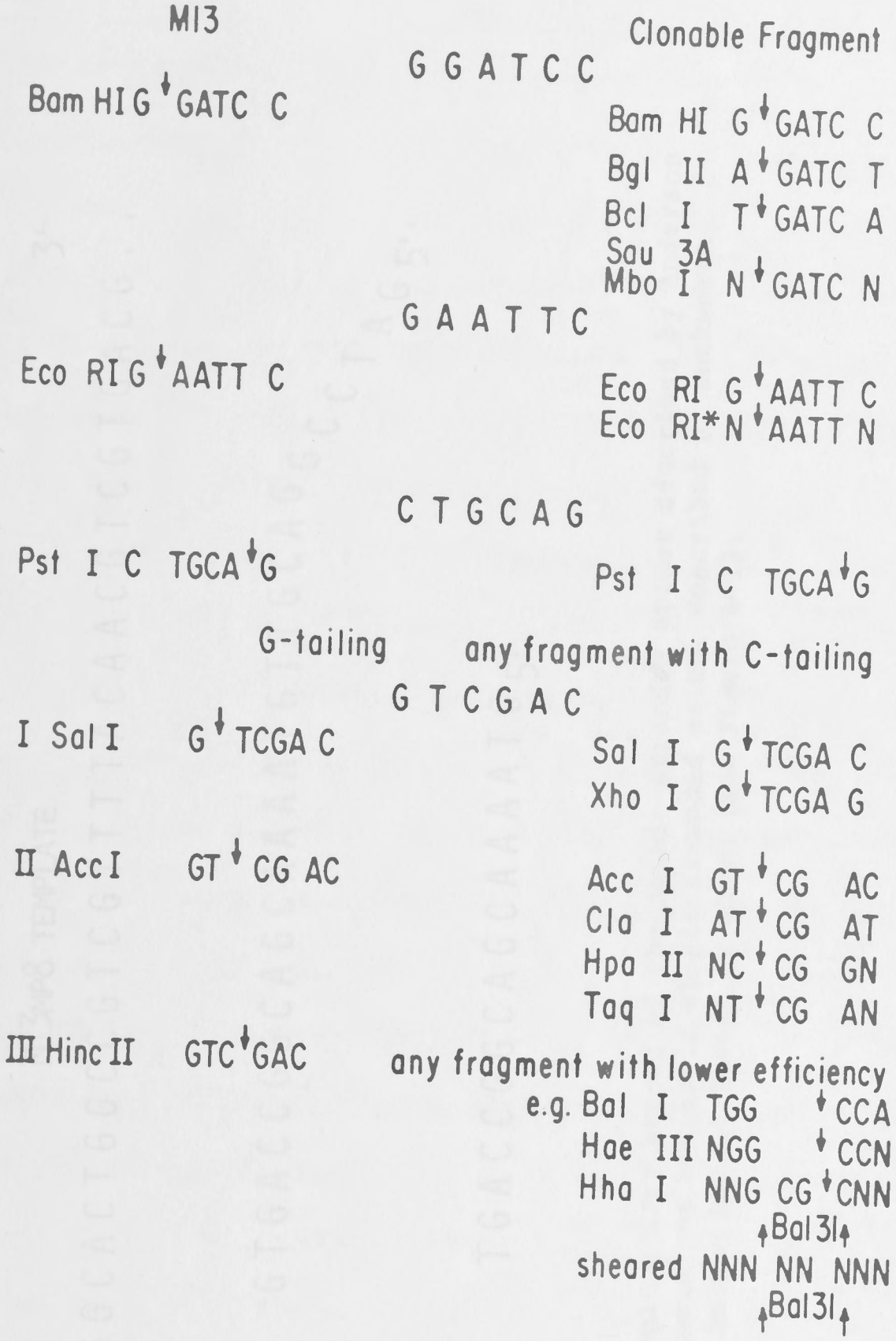
Two different universal primers were used for DNA sequencing of M13mp8 clones. These were the 26 bp double stranded primer of Anderson *et al.* (1980) and the 17-base synthetic primer described by Duckworth *et al.*, (1981). The primers and their sites of priming are shown in Figure 6-3. Both primers were kindly provided by Dr I.G.Young.

Figure 6-1. A Genetic Map of M13mp7, M13mp8 and M13mp9



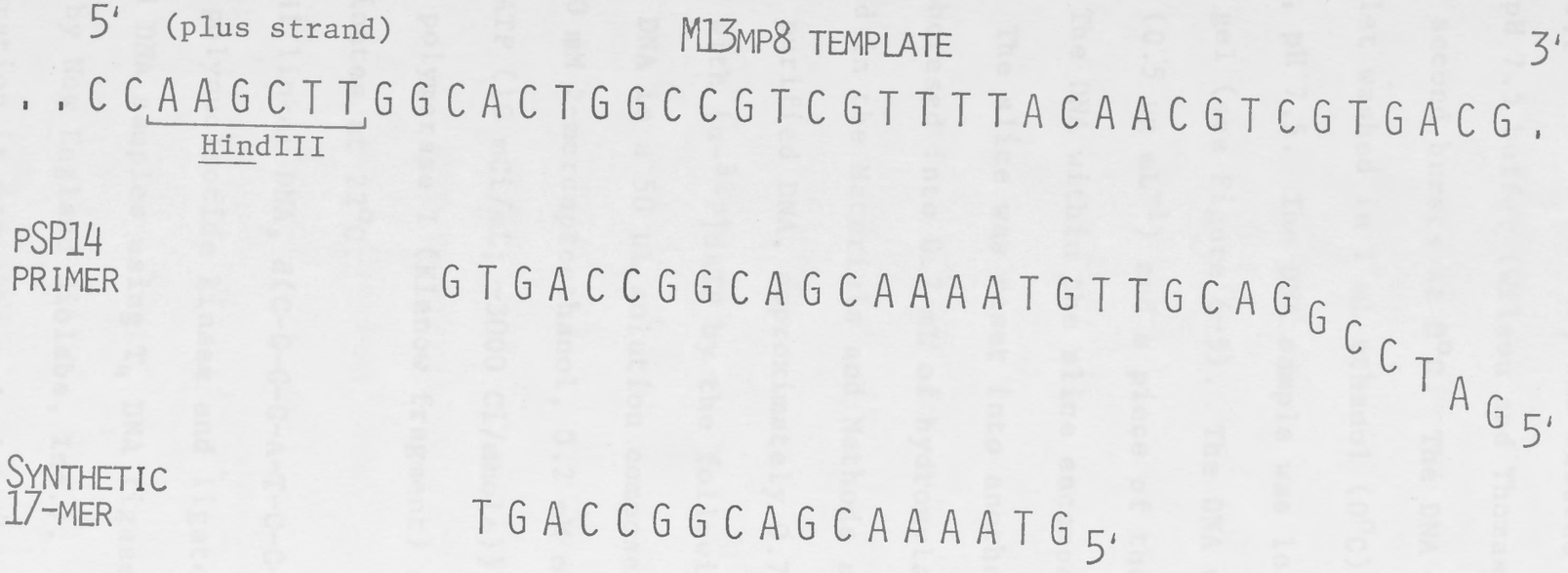
Map units are given in kb. The difference between the three molecules is given in detail by the nucleotide sequence of the pertinent region of the lacZ gene. The recognition sites for restriction endonucleases are labelled. The interruption of the wild-type amino acid sequence in M13mp2 by the inserted amino acid sequences is set up and numbered. The figure is taken from Messing and Vieira (1982).

Figure 6-2. Cloning Guide to M13mp7



The enzymes and their recognition sequences that are used for cleaving the vector are listed on the left side. The enzymes and treatments used to produce DNA fragments which may be cloned at each M13mp7 site are listed on the right side. Cutting patterns are indicated by arrows and sticky ends are set apart from flanking nucleotides. The figure is taken from Messing et al., (1981).

Figure 6-3. Primers for Sequencing of Cloned DNA in Phage M13mp8



The listed pSP14 primer is the complementary strand of the double-stranded primer described by Anderson et al., (1980). The 17-mer represents the synthetic single-stranded primer described by Duckworth et al., (1981). The HindIII site in the M13mp8 insert is shown (see Figure 6-1).

Sonication of Fragment 2-1 DNA, BamHI Linker Addition and Attempts to Clone the Dld gene into pBR322

2 µg of a preparation of fragment 2-1 (see Figure 6-4) in 50 µL of $T_{10}E_{0.1}$ buffer, pH 7.5, was diluted to 1 mL in $T_{10}E_{0.1}$, 2.5 M NaCl, pH 7.5 buffer (Wilson and Thomas, 1974) and sonicated twice with 10 second bursts at 0°C. The DNA was ethanol precipitated, the pellet washed in 1 mL ethanol (0°C) and dissolved in 100 µL $T_{10}E_{0.1}$, pH 7.5. The DNA sample was loaded into 4 lanes of an agarose gel (see Figure 6-5). The DNA was stained with ethidium bromide (0.5 µg mL^{-1}) and a piece of the gel was cut out with a razor blade. The DNA within the slice encompassed the size range of 1.8 to 4.0 kb. The slice was reset into another agarose gel and the DNA electrophoresed into 0.7 mL of hydroxylapatite and purified as described in the Materials and Methods section of Chapter 2.

The purified DNA, approximately 0.7 µg, was 'filled-in' and labelled with [α-^{32}P]dATP by the following procedure. To the purified DNA in a 50 µL solution composed of (60 mM Tris HCl, 8 mM $MgCl_2$, 10 mM 2-mercaptoethanol, 0.2 mM of dGTP, dTTP and dGTP and 2µL [α-^{32}P]dATP (10 mCi/mL; ~3000 Ci/mmole)) was added 0.3 units of E. coli DNA polymerase I (Klenow fragment) and the solution incubated for 30 minutes at 22°C.

BamHI linker DNA, d(C-G-G-G-A-T-C-C-C-G), was phosphorylated using T_4 polynucleotide kinase and ligated to the 'filled-in' sonicated DNA samples using T_4 DNA ligase (linker and enzymes were supplied by New England Biolabs, Inc.). After the ligation of the DNA preparation it was digested with BamHI restriction endonuclease (fragment 2-1 does not contain a BamHI site) and loaded onto a 5 mL Sephadex G-75 column equilibrated with 10 mM Tris HCl, 1 mM EDTA, 100 mM NaCl, pH 7.5 buffer. Column fractions were subjected to liquid

scintillation spectrometry and were pooled taking care not to include linker DNA fractions. The pooled column fractions were ethanol precipitated and dissolved in $T_{10}E_{0.1}$ buffer, pH 7.5.

2 µL (0.5 µg) BamHI digested pBR322 DNA and 3 µL of the sonicated DNA sample were mixed and additions were made to give a final buffer composition of (66 mM Tris HCl, pH 7.5, 60 mM $MgCl_2$, 50 µg mL^{-1} BSA, 10 mM dithiothreitol, 1 mM rATP). The mixture was incubated for 5 minutes at 37°C, 2 µL T_4 DNA ligase (200 units) was added and the incubation continued for 16 hours at 15°C. The DNA was transformed into strain IY12 as described in the Materials and Methods section of Chapter 2.

Preparation of BamHI and AccI digested M13mp8

To 50 µL M13mp8 (220 µg mL^{-1}) in an Eppendorf tube was added 37 µL sterile distilled water, 10 µL sterile buffer (1.5 M NaCl, 60 mM Tris HCl, pH 7.9, 60 mM $MgCl_2$, 1 mg mL^{-1} BSA) and 3 µL of BamHI endonuclease (15 units; New England, Biolabs, Inc.). The solutions were mixed and incubated for 1.5 hours at 37°C. The mixture was then vortexed (10 seconds) in the presence of 200 µL of neutralized phenol and the phases separated by centrifugation for 1 minute in an Eppendorf centrifuge. Residual phenol was removed from the aqueous phase by two extractions with 400 µL of diethyl ether. The DNA was ethanol precipitated by the addition of 0.1 volume of 5 M NaCl and 2.5 volumes of ethanol. The solution was mixed and placed into a cardice-ethanol bath for 1 hour. The DNA was pelleted by centrifugation for 10 minutes in an Eppendorf centrifuge. The supernatant was removed and the pellet (not visible) was washed with 1 mL of cold ethanol. The pellet was dried for 5 minutes *in vacuo*

and then dissolved in 100 µL $T_{10}E_{0.1}$ buffer, pH 7.5.

The same procedure was used for the preparation of the AccI digested M13mp8 except that the digestion buffer added was composed of: 60 mM NaCl, 60 mM Tris HCl, pH 7.5, 60 mM $MgCl_2$, 60 mM 2-mercaptoethanol, 1 mg mL^{-1} BSA.

Endonuclease Digestion of Purified 2-1 Fragment with Sau3A or HpaII

Plasmid pIY2 was digested with HindIII endonuclease and the cloned fragment 2-1 was purified according to the procedures outlined in the Materials and Methods section in Chapter 2.

To 50 µL of purified fragment 2-1 (3 µg), was added 5 µL buffer (500 mM NaCl, 60 mM Tris HCl, pH 7.5, 50 mM $MgCl_2$, 1 mg mL^{-1} BSA) and 1 µL (2 units) Sau3A endonuclease. The solutions were mixed and incubated at 37°C for 3 hours. After digestion the mixture was extracted with phenol and ether and then ethanol precipitated as described above.

The same procedure was used for the preparation of the HpaII digested fragment 2-1 except that the digestion buffer used was composed of: 60 mM KCl, 100 mM Tris HCl, pH 7.4, 100 mM $MgCl_2$, 10 mM dithiothreitol, 1 mg mL^{-1} BSA.

Ligation of Digested Fragment 2-1 into M13mp8

Fragments derived from the Sau3A or HpaII digestion of fragment 2-1 were cloned into BamHI and AccI digested preparations of M13mp8, respectively. The ligation procedure used was as follows: 1 µL (~100 ng) digested M13mp8 was added to 1 µL (~50 ng) digested

fragment 2-1, this mixture was then added to a solution composed of (1 µL 1 M Tris HCl, pH 7.5, 1 µL 1 M $MgCl_2$, 1 µL 1 mg mL^{-1} BSA, 1.5 µL 0.1 M dithiothreitol; 1.5 µL 10 mM rATP, 6 µL sterile distilled water). These solutions were mixed and then placed at 37°C for 5 minutes. 2 µL of T_4 DNA ligase (200 units) was added, mixed and placed at 15°C for 16 hours. After ligation, 5 µL of the mixture was used to transfect JM103 as described in the Materials and Methods section of Chapter 2.

Computer Programs Used for the Storage and Manipulation of DNA Gel Reading Data

The computer programs used in this study have been described by Staden (1977;1980). The files containing sequence data were stored on magnetic disks (Data cartridge RL02K-DC; Digital Equip. Corp., Maynard, Massachusetts). The computer used was a PDP 11/34 (Digital). The programs are all written in PDP FORTRAN using many small subroutines, some of which are common to all programs. The applications of the programs are listed below.

BATIN: This program is used to enter new gel reading data on the disk and is used for the initial imput of data. Each gel reading is given a file name and is stored in an individual disk file. The program also writes to another file (file of file names) which contains the names of all the gel reading files it has written during each run.

DBCOMP: This program is used to search for overlaps between sequences. It compares each gel reading file with each sequence file and then compares the gel reading files with each other. Any overlaps found (the operator defines the minimum length of overlap)

are sorted, their positions listed, printed and the complementary regions of the two overlapping sequences are displayed.

DBCOMP is also valuable in that it can compare sequence data obtained with the known sequence of M13mp2 and pBR322 (which shares some sequence with pGM706) and hence can detect any extraneous sequences derived from either vector.

DBUTIL: This program is used to establish and change the relationships between gel reading data. Relationships (ie which gels overlap with which) are set up and maintained in a special file which is called the database or file of relationships. It is this file that is used and, if necessary, updated by all the functions supplied by this program (Staden, 1980).

The DBUTIL program has a number of functions which makes it particularly versatile. A number of important features are outlined below.

(a) The enter function allows entry of a new gel reading into the database system after it has been scrutinized by the DBCOMP program.

(b) The entry of gel reading data expands the database, the contents of which can be readily printed using the print function. Furthermore, the contents of any region of the database can be displayed. The display function readily shows the operator any problem areas in the lined up sequences.

(c) The edit function allows the operator to edit any section of sequence.

(d) The complement function allows the operator to complement and reverse all the gels covering any contiguous section of sequence.

(e) As the gel reading data entered increases, various contigs expand in length. A join function allows overlapping contigs to be appropriately aligned and joined to make a larger composite contig. Ultimately the database will be composed of only one contig. Furthermore, the display function can be used to detect any regions which may require resequencing.

Hence, the DBUTIL program offers a number of significant advantages over manual sequence data processing or the use of simple computer storage and retrieval systems. These advantages include: (a) progress can be rapidly monitored (functions; print display), (b) ambiguities can be readily detected and edited (function edit), (c) overlapping contigs can be expanded to ultimately form a continuous composite (functions; join complement), and (d) the cumulative nature of the data collection process readily enables the operator to assess the reliability (on each DNA strand) of any section of the sequence.

RESULTS AND DISCUSSION

The D-lactate dehydrogenase has a molecular weight of approximately 63000 (calculated from data presented in Santos et al., 1982). The coding region of the dld gene would therefore be expected to be approximately 1600-1800 bases long. The size of the cloned fragment carrying the dld gene is 6.9 kb and therefore is considerably larger than the estimated size of the dld gene.

The standard approach would be to subclone fragments of the 6.9 kb cloned insert so as to obtain the dld gene on a smaller piece of

DNA. This approach was attempted, while at the same time the random sequencing method was used to initiate the sequencing of the whole fragment.

The sequencing approach used in this work involved the production of restriction fragments using enzymes with 4-base recognition sequences and the random cloning of these fragments into M13 vectors. This approach has been used extensively by Dr F. Sanger and his colleagues and has been found to be a convenient approach in rapidly obtaining sequence data (Sanger et al., 1980; 1982).

Isolation and Purification of the Cloned HindIII Fragment Containing the Dld gene

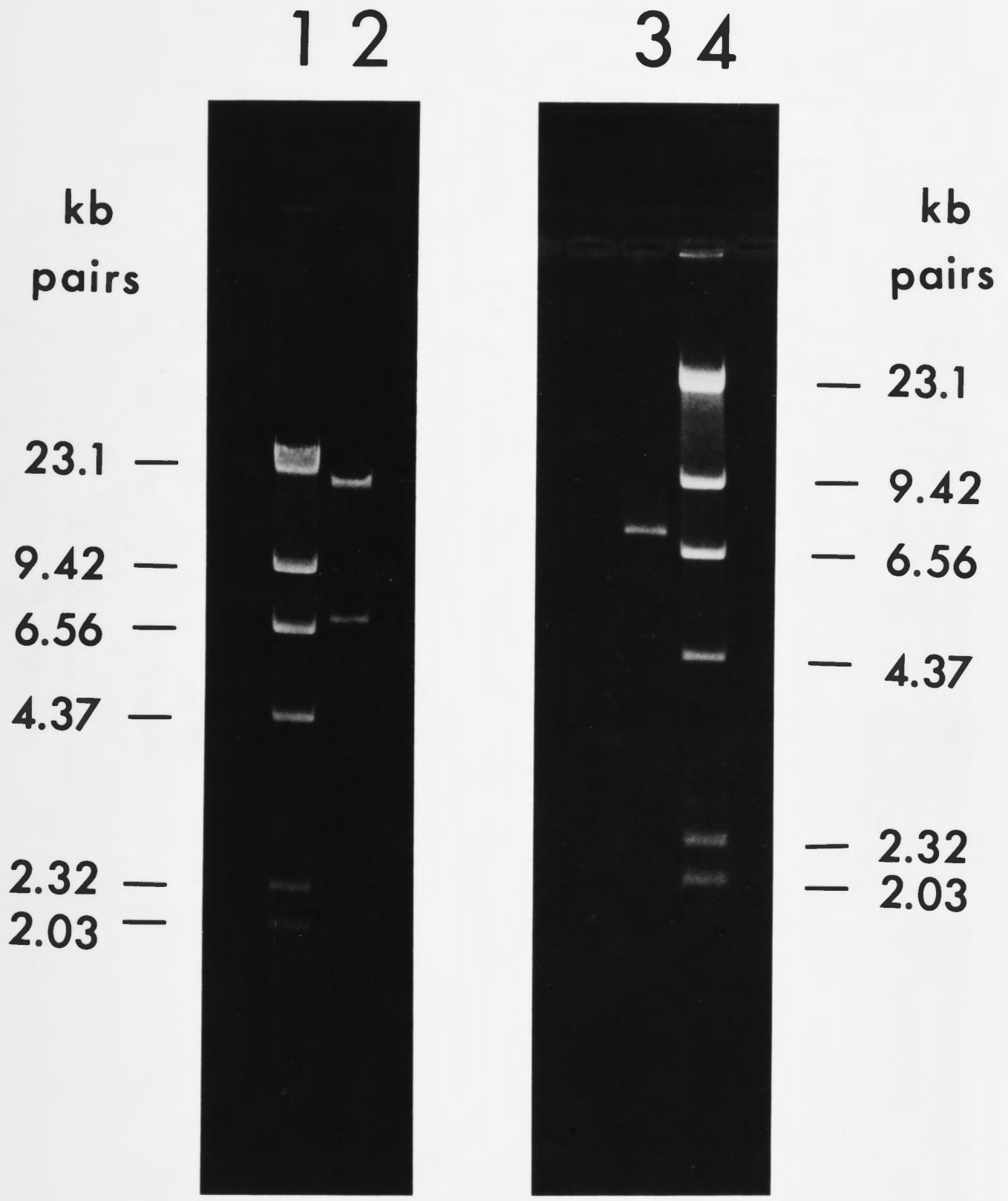
The plasmid pIY2 was isolated from strain IY36 by the method of Clewell and Helinski (1969) (see Materials and Methods Chapter 2). Plasmid pIY2 was then digested with HindIII and the cloned 4.6 Mdal fragment (designated 2-1) purified by agarose gel electrophoresis and hydroxylapatite chromatography (see Figure 6-4).

Attempts to Subclone the Dld Gene

The strategy used was to shorten the 6.9 kb fragment by sonication, add linkers and to clone into pBR322.

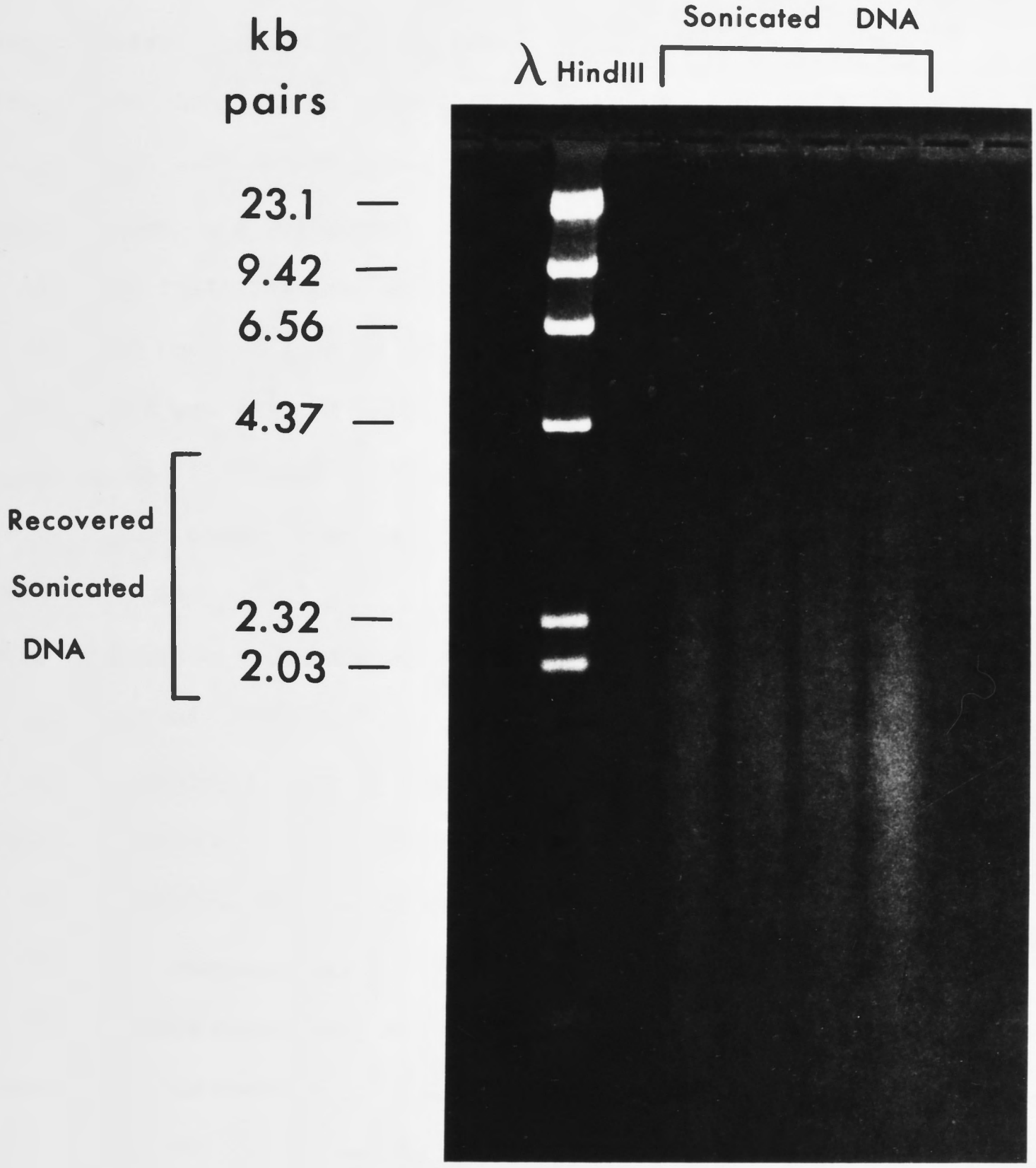
The purified 6.9 kb was sonicated and the DNA fragments produced were size fractionated on a preparative agarose gel (Figure 6-5). DNA fragments encompassing the size range of 1.8 to 4.0 kb were purified by hydroxylapatite chromatography as described in the Materials and Methods section of Chapter 2.

Figure 6-4. Electrophoresis of Plasmid DNA and Purified Fragment 2-1 on 0.8% (w/v) Agarose Gels



Samples : (1) and (4) HindIII restriction fragments of λcI857, (2) pIY2 digested with HindIII, and (3) Purified Fragment 2-1 (~6.9kb). DNA was digested, subjected to electrophoresis and purified as described in the Materials and Methods of Chapter 2.

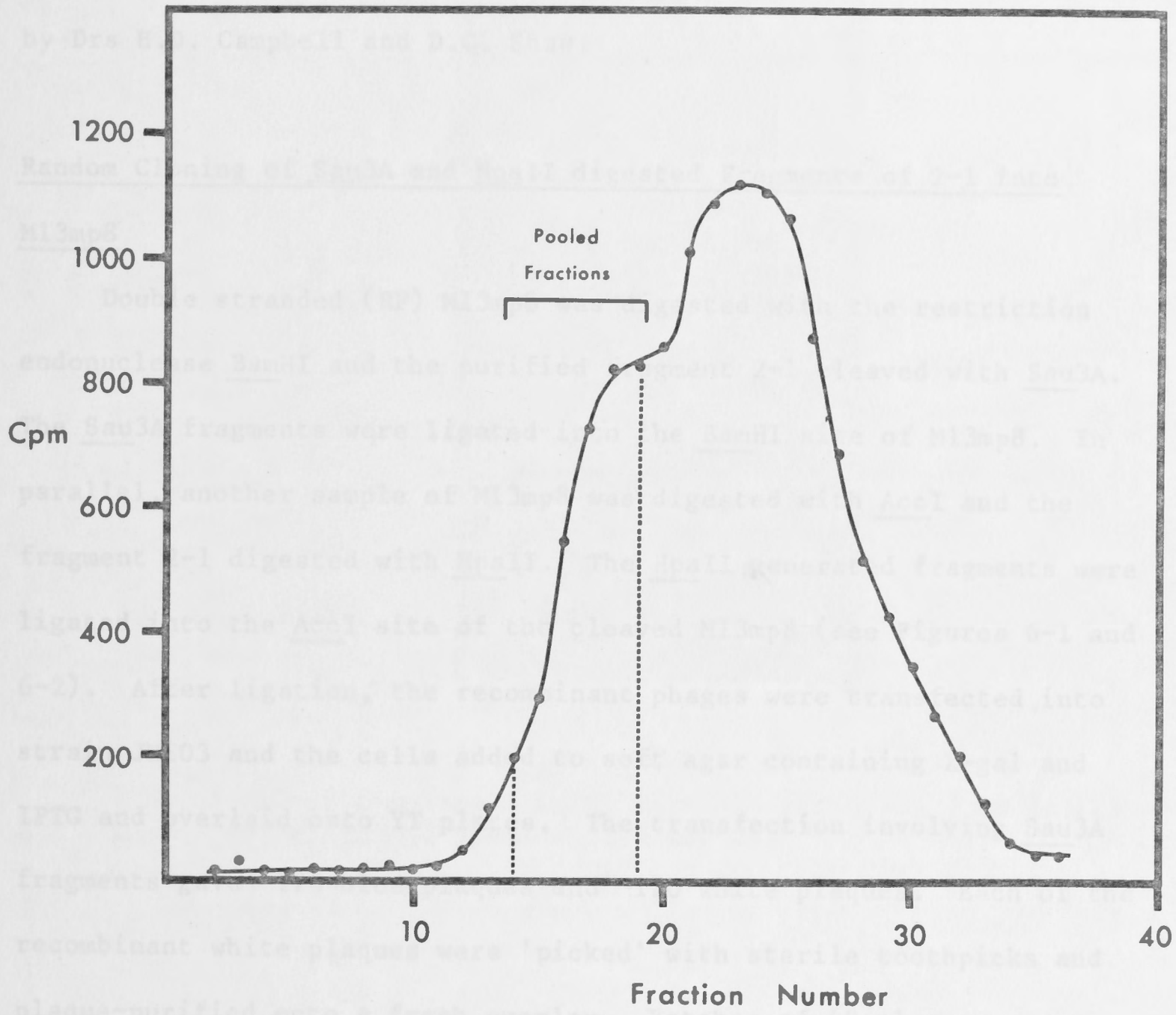
Figure 6-5. Sonication and Size Fractionation of Fragment 2-1 on a 0.8% (w/v) Agarose gel



The sonicated DNA, size range 1.8 to 4.0 kb, was purified as described in the Materials and Methods section.

The purified fractionated DNA samples had their ends 'filled-in' using dNTPs, [α-^{32}P]dATP, and Klenow polymerase enzyme. The 'filled-in' DNA samples were ligated to phosphorylated BamHI linkers. Each sample was then digested with BamHI endonuclease and fractionated on a Sephadex G-75 column to remove free linker (Figure 6-6). The first third of the peak fractions shown in Figure 6-6 were pooled and concentrated by ethanol precipitation. The BamHI linker ligated DNA was ligated into the BamHI site of pBR322 and transformed into strain IY12 (ndh). Transformants were screened by firstly selecting on complete media (brain heart infusion agar, BHIA) plus ampicillin (Ap; 25 μg mL^{-1}). Approximately 120 Ap^{R} transformants were replated onto BHIA plus tetracycline (Tc; 10 μg mL^{-1}). Eight of the 120 colonies were Tc^{S}, indicating that they contained inserts. It was necessary to see if any of these could complement the ndh mutant so each of these colonies was replated onto mannitol-minimal plates (Materials and Methods section, Chapter 4). None gave convincing complementation. It may be that the failure to obtain a hybrid plasmid containing a functional dld gene was due to the relatively low numbers obtained. However, the complementation of the ndh mutants by the cloned dld gene would be dependent on the level of amplification of the D-lactate dehydrogenase and for some reason this may not be as high with the present plasmids as with pIY2. Dld mutants are available in the E. coli ML strain but growth of the parent ML strain on D-lactate is poor. Thus, a simple subcloning strategy by complementation was not available. Although subcloned fragments could be screened by coupled transcription/translation or measurement of D-lactate dehydrogenase in membrane preparations, it was decided to use a random sequencing approach until the N-terminus

Figure 6-6. Sephadex G-75 Column Chromatography of Sonicated Fragments of 2-1 after BamHI Linker Addition



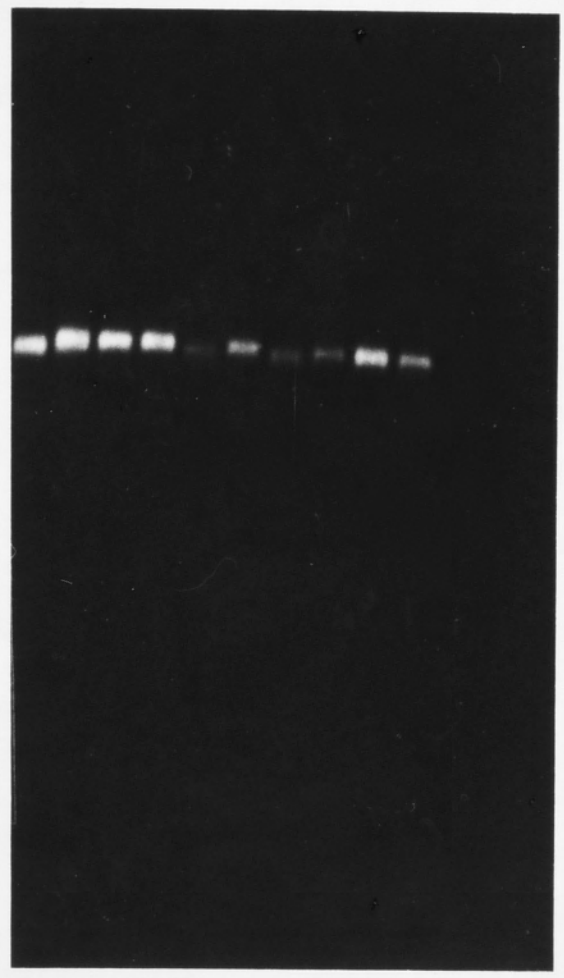
See text and the Materials and Methods section for further details.

of the dld coding region could be identified. The N-terminal sequence of the D-lactate dehydrogenase is currently being determined by Drs H.D. Campbell and D.C. Shaw.

Random Cloning of Sau3A and HpaII digested Fragments of 2-1 into M13mp8

Double stranded (RF) M13mp8 was digested with the restriction endonuclease BamHI and the purified fragment 2-1 cleaved with Sau3A. The Sau3A fragments were ligated into the BamHI site of M13mp8. In parallel, another sample of M13mp8 was digested with AccI and the fragment 2-1 digested with HpaII. The HpaII generated fragments were ligated into the AccI site of the cleaved M13mp8 (see Figures 6-1 and 6-2). After ligation, the recombinant phages were transfected into strain JM103 and the cells added to soft agar containing X-gal and IPTG and overlaid onto YT plates. The transfection involving Sau3A fragments gave ~120 blue plaques and ~170 white plaques. Each of the recombinant white plaques were 'picked' with sterile toothpicks and plaque-purified onto a fresh overlay. Batches of 40 plaques were then picked and grown up for template production. To examine whether the template production procedure had been successful, samples (5 µL volume) were electrophoresed on a 0.8% (w/v) agarose gel (Figure 6-7). It can be seen from Figure 6-7 that the DNA template yields were variable. The M13 clones carrying random short fragments were screened for their suitability as sequence templates by carrying out the normal sequencing reaction with only one dideoxynucleotide. In this case the positions of thymines in the DNA sequence were determined and used as a basis for comparison of the various clones. This procedure is referred to as 'T-tracking' and an

Figure 6-7. Electrophoresis of Recombinant M13mp8 DNA on a 0.8% (w/v) Agarose Gel



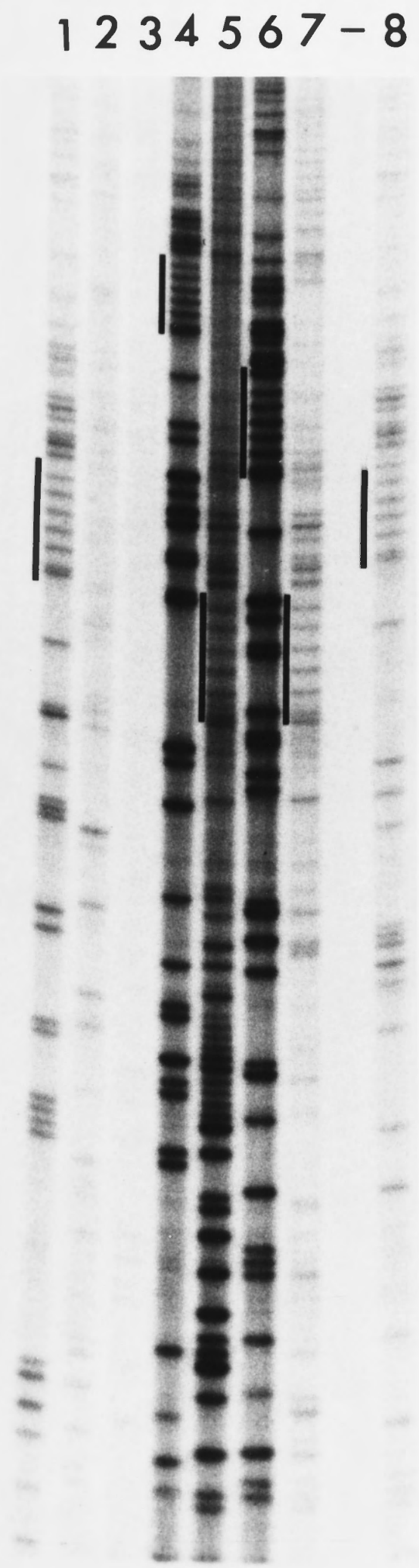
5 µL of M13mp8 template samples were electrophoresed as described in the Materials and Methods (Chapter 2).

example is provided in Figure 6-8. The variability in the template yields shown in Figure 6-7 was reflected in the intensity of the 'T-track' sequencing reactions shown in Figure 6-8, However, in the sequencing reactions the amount of template used was varied to compensate and hence this did not present a major difficulty. Furthermore, 'T-tracking' enables the size of the inserts to be determined by the recognition of the vector sequence (see Figure 6-8). Clones carrying the same insert can be identified and the suitability of clones for sequencing can be assessed. For example, the clone represented in lane 5 of Figure 6-8 appears to exhibit multiple banding and a high autoradiographic background and therefore would be rejected as a clone which is unsuitable for sequencing.

A number of the Sau3A and HpaII generated clones were sequenced. In most cases the reaction mix was electrophoresed on a 'long' and 'short' gel. Examples of sequence autoradiographs are shown in Figure 6-9. The autoradiographs presented in Figure 6-9 have been selected to highlight a number of characteristics of the sequencing gels. In Figure 6-9(a) it can be seen that the bands at the bottom of the gel are significantly less labelled than the bands in the upper region of the gel. This clone was sequenced using the 26 bp primer described by Anderson et al., (1980). When the 17-mer synthetic primer described by Duckworth et al. (1981) is used this difference is less pronounced, presumably due to greater label incorporation which makes reading of the gels in this region less difficult (see Figure 6-9, b,c and d). The use of universal primers gave considerably better sequence data than that obtained previously with internal primers (see Chapter 2).

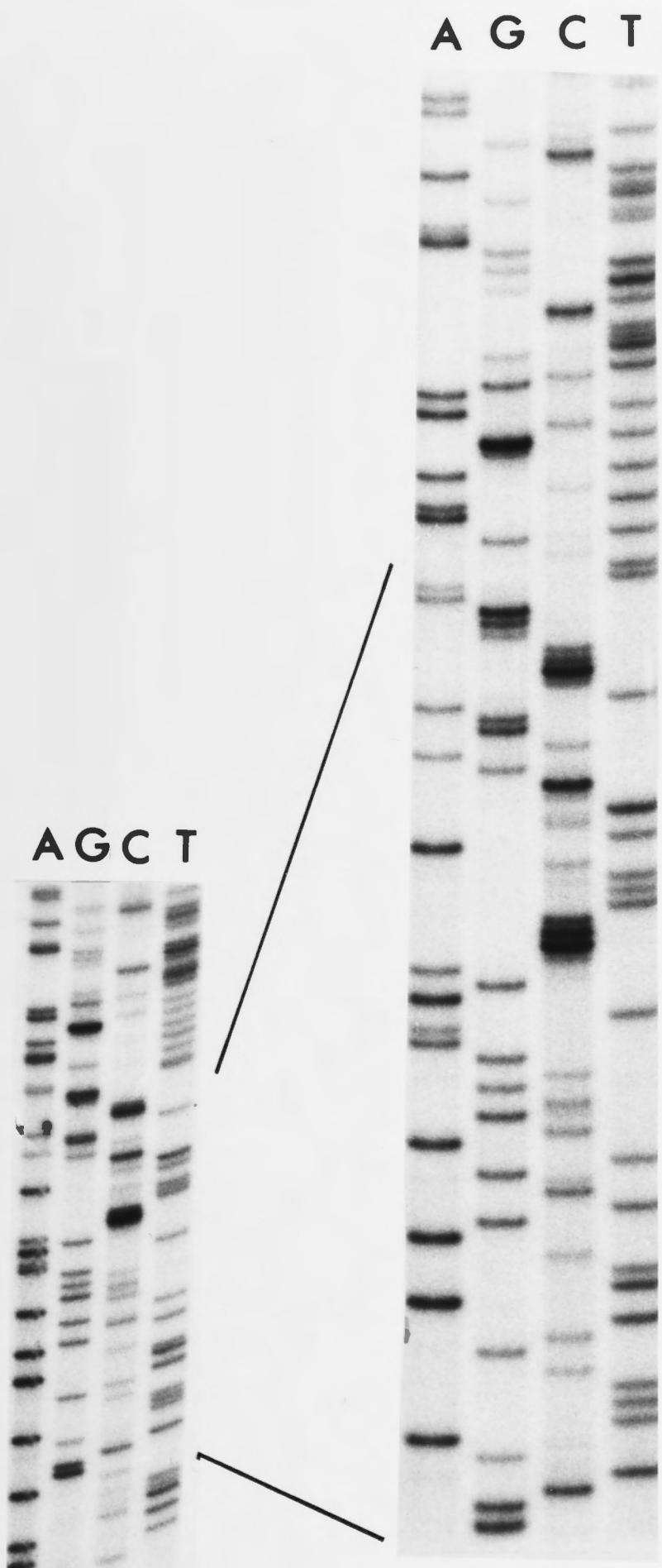
Some gels in Figure 6-9, especially those in the outer 'lanes' show a distortion of the bands which is attributed to heating effects

Figure 6-8. 'T-Tracks' of M13mp8 clones Carrying Random Short Fragments Derived from Fragment 2-1

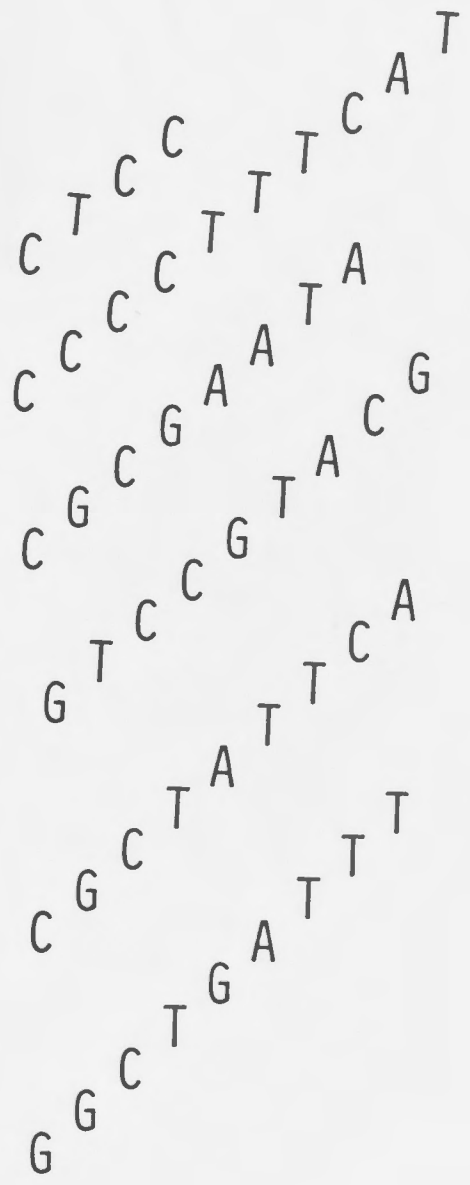


M13mp8 clones were prepared as described in the Materials and Methods section (Chapter 2). Each clone was used as a template in a 'T-lane' sequencing reaction. The vector sequence is illustrated by a vertical line. See text for further details.

Figure 6-9. Autoradiographs of DNA Sequencing Gels: The Determination of the Sequence of Cloned Sau3A and HpaII Fragments



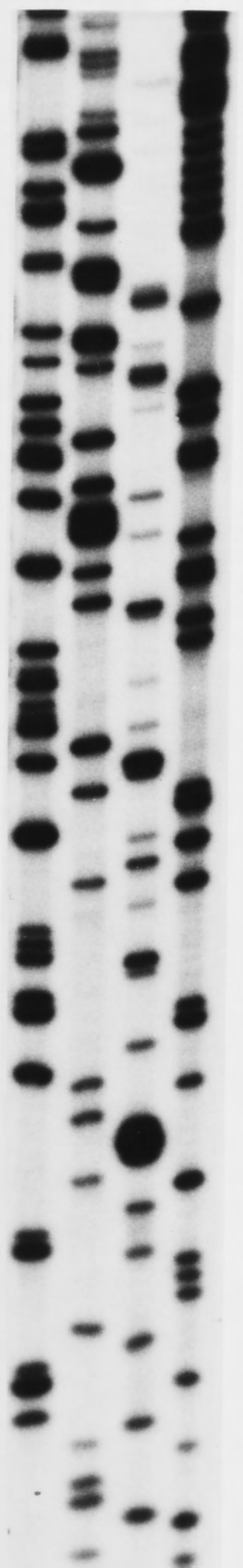
Long Gel

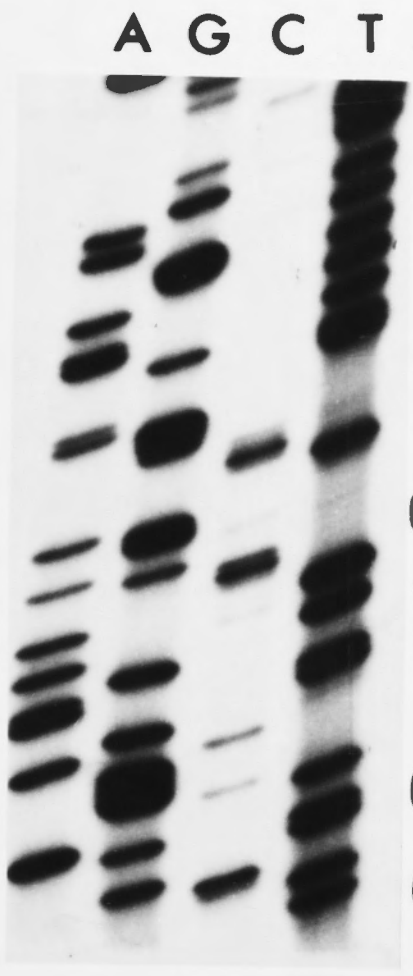


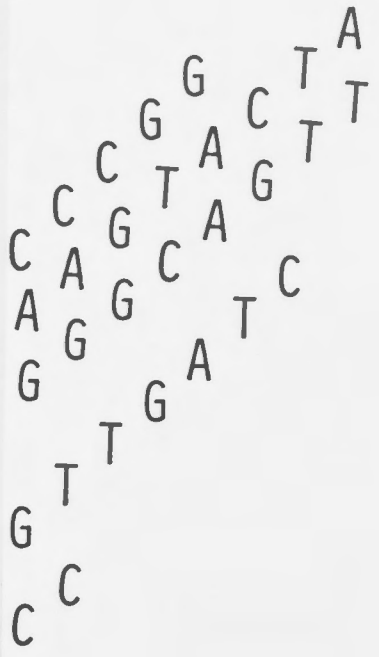
Gel (b)

Short Gel (HpaII Clones; 17-mer primer)

LDHLDH.004





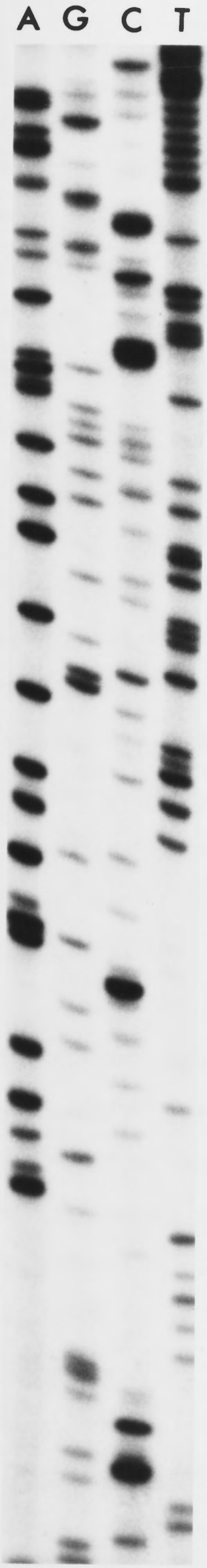


Gel (c)

Short Gel

(HpaII Clone; 17-mer primer)

LDHLDH.013



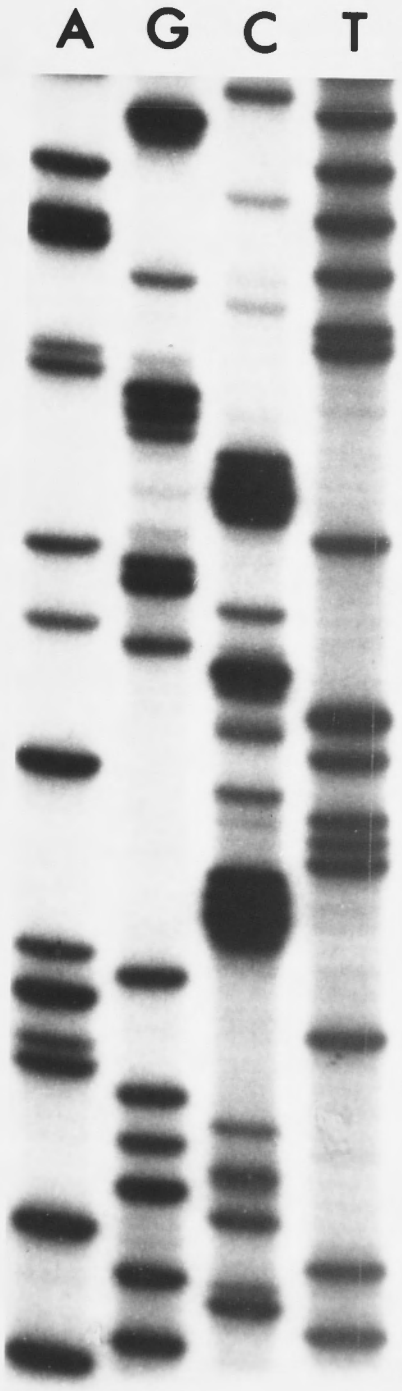
Gel (d)

Short Gel

Long Gel

(HpaII Clone; 17-mer primer)

LDHLDH.018



(ie the bands are not horizontal but are at an angle). This effect did not give rise to any difficulties in reading the gels. Some workers have used a thermostatic plate to overcome this problem (Garoff and Ansorge, 1981).

The main factor affecting the reading of the autoradiographs was the resolution of bands in the upper part of the gel. It was generally not possible to obtain readings longer than 120 nucleotides from the short gels, the limitation being band sharpness. The band sharpness in autoradiographs is affected by the quality of the sequencing gel itself and the method used for autoradiography. An improvement in the band sharpness with long gels was achieved by following the recommendation of Anderson (1981), ie using half strength TBE buffer. It has been suggested that the use of buffer gradient gels produces a more evenly-spaced band pattern and allows longer readings to be obtained (Amersham Research News, No. 27 Feb 1983). As yet the buffer gradient system has not been assessed in this laboratory.

Significant improvement in band resolution can also be obtained if the sequencing gels are dried before autoradiography (Sanger et al., 1982). Recently, it has also been demonstrated that the use of [α-^{35}S]dATPαS instead of [α-^{32}P]dATP in DNA sequencing also gives a significant improvement in band sharpness (Gilson, Barrell, unpublished results cited in Amersham Research News, No. 27 Feb 1983).

Perhaps one of the major advances in the speed of carrying out the sequencing reaction comes with the development of the microcentrifuge technique (Davies, 1982). This method, devised by Sanger and his colleagues, supersedes the methods which extensively

use glass capillaries as reaction vessels (Sanger et al., 1980). In the method described by Davies (1982), all the steps in the sequencing procedure are done in microcentrifuge tubes and mixing is achieved by a quick centrifugation step which allows ten reactions to be handled at a time. As pointed out by Davies (1982) the procedure involving the use of glass capillaries has the major disadvantages of requiring considerable time for the manipulations involved and it is difficult to handle more than four complete reactions at a time.

The Partial DNA Sequence of the Cloned Fragment 2-1 which contains the *Dld* Gene

Using the Staden (1980) computer program DBUTIL a DNA sequence database was constructed from the various gel readings. Sequence data was entered using the program BATIN and each reading was compared using DBCOMP (Staden, 1980) with the sequence of M13mp2 and pBR322 to eliminate any vector-derived clones. Gel readings were also compared with one another to detect any sequence overlaps. The construction of the database and the DNA sequence is shown in Figures 6-10 and 6-11, respectively. Approximately 1030 bases of unique DNA sequence is presented in Figure 6-11. Moreover, a number of contigs have been sequenced twice giving total internal agreement (see Figure 6-10 for DBUTIL printout).

Unfortunately, a high percentage of the *Sau*3A and *Hpa*II generated clones were relatively short (60-130 bp long). It has been observed previously that small fragments are more readily inserted into the vector M13mp2 (and presumably its derivatives) than large fragments (Sanger et al., 1980). The choice of other restriction endonucleases may have overcome this problem. Therefore, although

Figure 6-10. Processing of Sequence Data by Computer: Construction of a Sequence Database

After the relationships between gel readings are established by DBCOMP a sequence database is constructed using DBUTIL. Examples of the procedures used are shown below.

```
 R DBSTRT
INITIALIZE DATA BASE FILE
   PROJECT NAME = LDHLDH.DB1

STOP --

 R DBUTIL

    DBUTIL

     PROJECT NAME = LDHLDH.DB1

    NUMBER OF GELS = 0 NUMBER OF CONTIGS = 0
    FOR 120 CHARACTER LINES TYPE Y


    SELECT OPTION BY NUMBER
    STOP=0,ENTER=1,PRINT=2,DISPLAY=3,JOIN=4,COMPLEMENT=5,EDIT=6
     OPTION NUMBER = 1

    ENTER

     NAME OF ARCHIVE = LDH001
    WORKING NAME FOR THIS GEL = LDHLDH.001
    IF THIS GEL OVERLAPS TYPE Y
```

A series of unrelated gel readings (ie no overlaps as shown by DBCOMP) are entered first then overlapping complementary or homologous gel readings are entered.

When all entries are completed, sorted data is obtained as illustrated below.

```
SELECT OPTION BY NUMBER
STOP=0,ENTER=1,PRINT=2,DISPLAY=3,JOIN=4,COMPLEMENT-5,EDIT=6
 OPTION NUMBER = 2

NUMBER OF GELS = 18 NUMBER OF CONTIGS = 13
TO SELECT CONTIGS TYPE Y

FOR SORTED DATA TYPE Y

                987        93.          0         13         17
LDH019           13         1.         93          0         17
LDH020           17         1.         93         13          0

                988        43.          0         12         12
LDH018           12         1.         43          0          0

                989        87.          0         11         11
LDH017           11         1.         87          0          0

                990       114.          0         10         10
LDH016           10         1.        114          0          0

                991       111.          0          9          9
LDH015            9         1.        111          0          0

                992        72.          0          8          8
LDH014            8         1.         72          0          0

                993       128.          0          7          7
LDH013            7         1.        128          0          0

                994        58.          0          6          6
LDH012            6         1.         58          0          0

                995        23.          0          5          5
LDH011            5         1         23          0          0

                996        53.          0          4         18
LDH009            4         1.         53          0         18
LDH010           18         1.         53          4          0

                997        89.          0          3         14
LDH005            3         1.         89          0         14
LDH006           14         1.         86          3          0

                998        49.          0          2         15
LDH003            2         1.         49          0         15
LDH004           15         1.         49          2          0

                999       115.          0          1         16
LDH001            1         1.        115          0         16
LDH002           16         1.        115          1          0

SELECT OPTION BY NUMBER
STOP=0,ENTER=1,PRINT=2,DISPLAY=3,JOIN=4,COMPLEMENT-5,EDIT=6
 OPTION NUMBER = 0
```

The first column lists the names of the archives (gel reading names; eg LDH001) grouped into their respective contigs. The second column lists the numbers of the gel working names (numbers 1-18; eg LDHLDH.001). The third and fourth columns list the length of DNA sequence covered on each sequence strand. Columns five and six list the gel working names and shows which gel readings are complementary or homologous and on which DNA strand.

One major advantage of the DBUTIL program is that the operator can readily assess the reliability of entered sequence data. This is done using the display option. Each gel reading sequence is automatically compared and a consensus sequence is listed. This is shown in the examples below.

```
R DBUTIL

   DBUTIL

    PROJECT NAME = LDHLDH.DB1

   NUMBER OF GELS = 18 NUMBER OF CONTIGS = 13
   FOR 120 CHARACTER LINES TYPE Y


SELECT OPTION BY NUMBER
STOP=0,ENTER=1,PRINT=2,DISPLAY=3,JOIN=4,COMPLEMENT-5,EDIT=6
 OPTION NUMBER = 3
 NUMBER OF LEFT GEL THIS CONTIG = 1
DEFINE REGION
 RELATIVE POSITION OF LEFT END =
 RELATIVE POSITION OF RIGHT END =

            10.        20.        30.        40.        50.        60.
 1  GATCGCATCAAAGATGACGATGTGCGTCACGATGGTCGTCACGCCCACGATTATGACTAT
16  GATCGCATCAAAGATGACGATGTGCGTCACGATGGTCGTCACGCCCACGATTATGACTAT
    GATCGCATCAAAGATGACGATGTGCGTCACGATGGTCGTCACGCCCACGATTATGACTAT

            70.        80.        90.       100.       110.       120.
 1  GTCCACCGCGTTCGTGATATTGAAGCCGAXACGCCCGCACGTTATAACGCCGATC
16  GTCCACCGCGTTCGTGATATTGAAGCCGACACGCCCGCACGTTATAACGCCGATC
    GTCCACCGCGTTCGTGATATTGAAGCCGACACGCCCGCACGTTATAACGCCGATC

SELECT OPTION BY NUMBER
STOP=0,ENTER=1,PRINT=2,DISPLAY=3,JOIN=4,COMPLEMENT=5,EDIT=6
 OPTION NUMBER = 3
 NUMBER OF LEFT GEL THIS CONTIG = 2
DEFINE REGION
 RELATIVE POSITION OF LEFT END =
 RELATIVE POSITION OF RIGHT END =

            10.        20.        30.        40.        50.
 2  GATCTCCATACACTTATCTCACAATATGGTTATGCCGCGCCTGGTGATC
15  GATCTCAATACACTTATCTCACAATATGGTTATGCCGCGCCTGGTGATC
    GATCTCAATACACTTATCTCACAATATGGTTATGCCGCGCCTGGTGATC
```

```
SELECT OPTION BY NUMBER
STOP=0,ENTER=1,PRINT=2,DISPLAY=3,JOIN=4,COMPLEMENT=5,EDIT=6
 OPTION NUMBER = 3
 NUMBER OF LEFT GEL THIS CONTIG = 3
DEFINE REGION
 RELATIVE POSITION OF LEFT END =
 RELATIVE POSITION OF RIGHT END =

          10.       20.       30.       40.       50.       60.
 3  GATCGCGATGTCGTTATCATCAGCACCCTGCGTCTCGACAAGCTGCACGTTCTTGGCAAG
14  GATCGCGATGTCGTTATCATCAGCACCCTGCGTCTCGACAAGCTGCACGTTCTTGGCAAG
    GATCGCGATGTCGTTATCATCAGCACCCTGCGTCTCGACAAGCTGCACGTTCTTGGCAAG

          70.       80.       90.      100.      110.      120.
 3  GGCGAACAGGTGCTGGCCTATCCGGGCAC
14  GGCGAACAGGTGCTGGCCTATCCGGG
    GGCGAACAGGTGCTGGCCTATCCGGGCAC

SELECT OPTION BY NUMBER
STOP=0,ENTER=1,PRINT=2,DISPLAY=3,JOIN=4,COMPLEMENT=5,EDIT=6
 OPTION NUMBER = 3
 NUMBER OF LEFT GEL THIS CONTIG = 4
DEFINE REGION
 RELATIVE POSITION OF LEFT END =
 RELATIVE POSITION OF RIGHT END =

          10.       20.       30.       40.       50.       60.
 4  GGCTGATTTCGCTATTCAGTCCGTACGCGCGAATAGACCCCTTTCATCTCCGG
18  GGCTGATTTCGCTATTCAGTCCGTACGCGCGAATAGACCCCTTTCATCTCCGG
    GGCTGATTTCGCTATTCAGTCCGTACGCGCGAATAGACCCCTTTCATCTCCGG
```

```
SELECT OPTION BY NUMBER
STOP=0,ENTER=1,PRINT=2,DISPLAY=3,JOIN=4,COMPLEMENT=5,EDIT=6
 OPTION NUMBER = 3
 NUMBER OF LEFT GEL THIS CONTIG = 13
DEFINE REGION
 RELATIVE POSITION OF LEFT END =
 RELATIVE POSITION OF RIGHT END =

          10.       20.       30.       40.       50.       60.
13  CCGGTGCATAACGTTTCAACTGCCCGTGACTTAACCAAACTGCTCATTGCCAGCAAACAA
17  CCGGTGCATAACGTTTCAACTGCCCGTGACTTAACCAAACTGCTCATTGCCAGCAAACAA
    CCGGTGCATAACGTTTCAACTGCCCGTGACTTAACCAAACTGCTCATTGCCAGCAAACAA

          70.       80.       90.      100.      110.      120.
13  TATCCGTTGATCGGGCAGTTAAGTACTACCGG
17  TATCCGTTGATCGGGCAGTTAAGTACTACCGG
    TATCCGTTGATCGGGCAGTTAAGTACTACCGG

SELECT OPTION BY NUMBER
STOP=0,ENTER=1,PRINT=2,DISPLAY=3,JOIN=4,COMPLEMENT=5,EDIT=6
 OPTION NUMBER = 0
```

Figure 6-11. The Partial DNA Sequence of the Cloned Fragment 2-1 which contains the Dld Gene

A consensus file was constructed from the data accumulated in the database.

```
.R CONSEN

   CONSEN

    PROJECT NAME = LDHLDH.DB1

   NUMBER OF GELS = 18 NUMBER OF CONTIGS = 13
    NAME FOR CONSENSUS FILE = LDHCN1
   TO SELECT CONTIGS TYPE Y

   TO CALC ANOTHER CONSENSUS TYPE Y
```

When the sequence is complete the database and the consensus sequence will be comprised of only one continuous contig. At present the database has 13 discontinuous contigs as is shown in Figure 6-10. The consensus sequence is listed below.

```
.R SEQLST

   PLEASE TYPE NAME OF FILE 1

LDHCN1

FIRST SEQ NO =
LAST SEQ  NO =

   1 OR 2 STRANDED OUTPUT?  TYPE NOW

   NUMBER OF GAPS BETWEEN LINES =

   FOR 120 LINE OUTPUT TYPE Y
```

```
        10         20         30         40         50         60
<---LDHLDH .013-----> CCGGTGCATA ACGTTTCAAC TGCCCGTGAC TTAACCAAAC

        70         80         90        100        110        120
TGCTCATTGC CAGCAAACAA TATCCGTTGA TCGGGCAGTT AAGTACTACC CGG<---LDH

       130        140        150        160        170        180
LDH.012--- -->CCGGTTT ATTATTGATA ACCGTACGCA TCACCAGACA ATGGCC<---

       190        200        210        220        230        240
LDHLDH.011 ----->GATC ATCGTGTATA GGCGCATCGG TCATCGGCGG TATTTGTAAC

       250        260        270        280        290        300
AACTCCGGCG GCCTCGCTGG TGCAACGTGG CCCGGCGTAT ACC<---LDH LDH.010---

       310        320        330        340        350        360
-->CCGGATC GTTCTCGCGA TAGAACTTCT GCAACGTCTC CGGGTTCTGC CGCCGTGCCA

       370        380        390        400        410        420
TGTTTAATCA GCTCTTTGAT TGCACGTGAT CGGAAACGGT GATGCCTTTA AAGCCCA<--

       430        440        450        460        470        480
-LDHLDH.00 9----->GAT CGCCACGCCA ATGAATGGCA TGGATGACAG CGACGTGAAG

       490        500        510        520        530        540
CCCGACGCGG AGCCTTCGAT TCCCTTGCGG CGTTTTGGCG AACGATGAGA TTGCCAGCTG
```

```
      550         560         570         580         590         600
TGTTGCTT<- --LDHLDH.0 08----->GA TCCTGCATGG CGCGGATATC CTGACGGGTT
      610         620         630         640         650         660
ACGGTGTTGA AAATCGCCCC AACCTGACCG TTTTTTGATC <---LDHLDH .007----->
      670         680         690         700         710         720
GATCGGCAAA GAGTGCGCGT TATTACTGGC GCCAGGGGTT TGATATTGGT ATTACCTGGC
      730         740         750         760         770         780
ACTCAGATGA AGAAGGGGCA AAAGATACCG CGCGTGAGGT AGTTAGCCAC GGCGTACGTG
      790         800         810         820         830         840
CGGAGATC<- --LDHLDH.0 06----->CC GGCAGATTTT CGAAGTTAGC CAGAATATGA
      850         860         870         880         890         900
CGGCGGATTT CGGTCAGCAC TTCCGG<--- LDHLDH.005 ----->GATC TATTCGAACC
      910         920         930         940         950         960
ACCCGGATC <---LDHLDH. 004----->G GCTGATTTCG CTATTCAGTC CGTACGCGCG
      970         980         990        1000        1010        1020
AATAGACCCC TTTCATCTCC GG<---LDHL DH.003---- ->GATCGCGA TGTCGTTATC
     1030        1040        1050        1060        1070        1080
ATCAGCACCC TGCGTCTCGA CAAGCTGCAC GTTCTTGGCA AGGGCGAACA GGTGCTGGCC
     1090        1100        1110        1120        1130        1140
TATCCGGGCA C<---LDHLD H.002----- >GATCTCAAT ACACTTATCT CACAATATGG
     1150        1160        1170        1180        1190        1200
TTATGCCGCG  CCTGGTGATC <---LDHLDH .001-----> GATCGCATCA AAGATGACGA
     1210        1220        1230        1240        1250        1260
TGTGCGTCAC GATGGTCGTC ACGCCCACGA TTATGACTAT GTCCACCGCG TTCGTGATAT
     1270        1280        1290           5          15          25
TGAAGCCGAC ACGCCCGCAC GTTATAACGC CGATC
```

very convenient, the cloning of restriction fragments can result in a relatively high proportion of short clones.

A solution to the problem of endonuclease generated short clones is to generate larger fragments by techniques such as shearing (Wilson and Thomas, 1974; Fuhrman, Deininger, LaPorte, Friedmann and Geiduschek, 1981) or DNase treatment (Anderson, 1981) followed by size fractionation.

Time did not permit the application of these techniques to the present problem but further sequencing of the dld fragment has been carried out in this laboratory by Dr H.D. Campbell using cloned DNA generated by sonication. These fragments (approximately 500 bp in length) were made blunt ended and cloned into the SmaI site of M13mp9. This has proved to be a much more efficient sequencing strategy allowing gel readings of around 300-450 bases for each clone.

SUMMARY

The cloned ~6.9 kb fragment (designated 2-1) which contains the dld gene was isolated and purified after HindIII digestion of pIY2. The fragment was sonicated to reduce its length to the size range of 1.8 to 4.0 kb. BamHI linkers were added to the purified fragments and attempts were made to clone these fragments into the BamHI site of pBR322. However, difficulties were encountered in the attempt to obtain a recombinant plasmid capable of complementing the ndh mutant (ie growth on mannitol-minimal plates).

The determination of the DNA sequence of the complete 2-1 fragment which contains the dld gene was initiated. Sau3A and HpaII

restriction endonucleases were used to cleave fragment 2-1 and the generated fragments were randomly cloned into the BamHI and AccI sites of M13mp8. The M13mp8 clones were isolated and a number were sequenced by the Sanger chain-terminating method using the 'universal' 26 bp and 17-base primers described by Anderson et al. (1980) and Duckworth et al., (1981). Approximately 1030 bases of unique DNA sequence was obtained.

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